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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this proposal is to further understand the molecular mechanisms of β-lap-induced apoptosis. We examined alterations in intracellular Ca^{2+} homeostasis using NQO1-expressing MCF-7 cells. β-Lap-exposed MCF-7 cells exhibited an early increase in intracellular Ca^{2+}, from endoplasmic reticulum Ca^{2+} stores. BAPTA-AM, an intracellular Ca^{2+} chelator, blocked early increases in Ca^{2+} levels and inhibited β-lap-mediated mitochondrial membrane depolarization, intracellular ATP depletion, specific and unique substrate proteolysis, and apoptosis. The extracellular Ca^{2+} chelator, EGTA, inhibited later apoptotic endpoints (observed > 8 h, e.g., substrate proteolysis and DNA fragmentation), suggesting that later execution events were triggered by Ca^{2+} influxes from the extracellular milieu. Collectively, these data suggest a critical, but not sole, role for Ca^{2+} in the NQO1-dependent cell death pathway initiated by β-lap.

Further work was directed at elucidating the execution phase of the apoptotic pathway induced by β-lap. We demonstrated that β-lap mediated a unique proteolytic apoptotic pathway in NQO1-expressing cells via μ-calpain activation, and upon activation, μ-calpain translocated to the nucleus. The apoptotic events in NQO1-expressing cells in response to β-lap were significantly delayed and survival enhanced via exogenous expression of calpastatin. Furthermore, we showed that μ-calpain cleaved PARP to a unique fragment (~60 kDa) different from that previously reported for calpains. We also provide evidence that β-lap-induced, μ-calpain stimulated, apoptosis does not involve any of the known caspases.

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Introduction

Understanding and exploiting cell death processes in various human breast cancer cells is a major focus of breast cancer research. The promise is that better understanding of apoptotic and anti-apoptotic processes will allow for improved anti-breast cancer efficacy of existing therapeutic agents, as well as the development of additional efficacious drugs which elicit programmed cell death during treatment, without causing complicating inflammation reactions in normal tissues.

Apoptotic processes occurring in breast cancer cells, particularly non-caspase-mediated cell death pathways, are poorly understood. Since many cancers are thought to form, in part, because of deficient caspase pathway expression or activation, novel ways of activating non-caspase-mediated pathways may be important for treating breast cancers. β-Lapachone (β-Lap) is a naturally occurring quinone present in the bark of the South American Lapacho tree. It has antitumor activity against a variety of human cancers, including colon, prostate, promyelocytic leukemia, and breast (1-3). We recently demonstrated that β-lap kills human breast and prostate cancer cells by apoptosis, a cytotoxic response significantly enhanced by NAD(P)H:quinone oxidoreductase (NQO1, E.C. 1.6.99.2) enzymatic activity (4,5). Apoptosis following β-lap administration was unique, in that an ~60 kDa PARP cleavage fragment, as well as distinct intracellular proteolytic cleavage of p53, were observed in NQO1-expressing breast or prostate cancer cells (4-6). We previously characterized the activation of a novel cysteine protease in various breast cancer cell lines with properties similar to the Ca^{2+}-dependent cysteine protease, calpain, after exposure to β-lap (6).

Calpains are a family of cysteine proteases existing primarily in two forms designated by the Ca^{2+} concentration needed for activation *in vitro,* μ- calpain (calpain-I) that requires micro-

molar amounts of Ca^{2+} and m-calpain (calpain-II) that requires milli-molar amounts of Ca^{2+}. Each form is a heterodimer consisting of a large catalytic and a small regulatory subunit that is activated by increased Ca^{2+} concentrations. Calpains are predominantly located in the cytoplasm (7,8), but can translocate to cellular membranes where they appear to become activated (9). Calpains have been implicated in neutrophil apoptosis, glucocorticoid-induced thymocyte apoptosis, and adipocyte differentiation, but their patterns of activation are not well characterized (10-12).

Ca^{2+} is recognized as an important regulator of apoptosis (13-17). Ca^{2+} may act as a signal for apoptosis by directly activating key proapoptotic enzymes (e.g., calpain); however, these proteolytic responses are poorly understood. The role of Ca^{2+} in cell death processes involving caspase activation has been examined in detail (18-21); however, the role of Ca^{2+} in non-caspase-dependent cell death responses is relatively unexplored. Thus, further understanding the role of Ca^{2+} and calpains in apoptosis may allow for modulation of current breast cancer treatments, as well as allow for the development of new and improved therapies.

Our hypothesis is that β-lap induces apoptosis through NQO1-mediated changes in intracellular Ca^{2+} homeostasis that result in the activation of a novel-calpain-mediated proteolytic pathway. Using NQO1-expressing breast cancer cells, we demonstrated that β-lap mediates a unique proteolytic apoptotic pathway via changes in intracellular Ca^{2+} homeostasis and μ-calpain activation. We demonstrated that increases in intracellular Ca^{2+} levels were critical for the apoptotic pathway induced by β-lap (22). Increased cytosolic Ca^{2+}, due to ER Ca^{2+} pool depletion, led to loss of mitochondrial membrane potential, ATP depletion, specific and unique substrate proteolysis, DNA fragmentation and cell death by apoptosis (22). We also demonstrated that upon activation, μ-calpain translocated to the nucleus where it can

proteolytically cleave PARP and p53. The other ubiquitously expressed calpain, m-calpain, does not appear to be activated in NQO1-expressing breast cancer cells after exposure to β-lap. We provide evidence that suggests that β-lap-induced, μ-calpain stimulated, apoptosis did not involve any of the known caspases. Furthermore, the apoptotic responses in NQO1-expressing cells to β-lap can be significantly delayed and survival enhanced via exogenous expression of calpastatin. Thus, suggesting a role for μ-calpain in β-lap-mediated apoptosis that is independent of caspase-activation.

Body

Objective: *To further understand the molecular mechanisms of β-lap-induced apoptosis and its ability to selectively target cancer cells. Understanding the molecular mechanisms involved in β-lap-mediated apoptosis could provide a rationale for future drug development and/or result in the modification of existing agents for use in treating breast cancer. The drug will also be used to elucidate a novel calpain-mediated apoptotic pathway.*

AIM 1

The goal of Aim 1 was to determine the effect of β-lap on intracellular Ca^{2+} homeostasis and apoptosis.

Accomplishment of Stated Tasks for Aim 1:

We completed all tasks for specific aim 1. We found that NQO1-expressing breast cancer cells exhibit an increase in intracellular Ca^{2+} levels from 4-9 min after β-lap exposure (Task 1) (Fig. 3 A, Tagliarino *et al., J. Biol. Chem.,* Appendix). Co-loading cells with the

intracellular Ca^{2+} chelator, BAPTA-AM, as well as the NQO1-specific inhibitor, dicumarol prevented intracellular Ca^{2+} increases after β-lap (Figs. 3 B and 7 A, Tagliarino *et al., J. Biol. Chem.,* Appendix). Therefore, the initial rise in Ca^{2+} was dependent upon the bioactivation of β-lap by NQO1. We also determined that the initial rise in intracellular Ca^{2+} was due to depletion of the endoplasmic reticulum (ER) Ca^{2+} stores. This was found by sequentially treating cells with β-lap and thapsigargin, a known agent that depletes ER Ca^{2+} stores. Both agents elicited a similar increase in intracellular Ca^{2+} that, when given first, prevented the other agent from eliciting a further increase in Ca^{2+} in those same cells (Figs. 3 C and D, Tagliarino *et al., J. Biol. Chem.,* Appendix). Therefore, it was unnecessary to transfect cells with cameleons to determine the pool of Ca^{2+} mobilized by β-lap addition, since it was determined that the pool of Ca^{2+} mobilized was that of ER Ca^{2+} stores (Task 2).

Utilizing the intracellular Ca^{2+} chelator, BAPTA-AM, and the extracellular Ca^{2+} chelator, EGTA, we were able to determine the effects of Ca^{2+} on downstream signaling events after bioactivation of β-lap by NQO1 (Task 3). We found that the initial increase in intracellular Ca^{2+} levels was at least partially responsible for ALL downstream events, including mitochondrial membrane depolarization, ATP depletion, specific and unique substrate proteolysis, and DNA fragmentation (Figs. 1, 4, 5, and 6, Tagliarino *et al., J. Biol. Chem.,* Appendix). We also found that Ca^{2+} from the extracellular milieu was needed for substrate proteolysis and DNA fragmentation (Fig. 1, Tagliarino *et al., J. Biol. Chem.,* Appendix, and (6). As expected, dicumarol pretreatment of MCF-7 cells prevented all β-lap mediated apoptotic events (Fig. 7, Tagliarino *et al., J. Biol. Chem.,* Appendix).

The cell death pathway induced by β-lap was quite distinct from that observed after exposure to TG, and β-lap-mediated apoptosis exhibited many similarities to menadione-

mediated apoptosis when high doses of menadione were used (>20 μM), or when co-administration of dicumarol was given (as discussed in Tagliarino *et al., J. Biol. Chem.,* Appendix). These observations further suggest that the early release of Ca^{2+} from ER stores, as well as influx of Ca^{2+} from the extracellular milieu, are necessary but not sufficient for the novel apoptotic pathway induced by β-lap. Thus, changes in Ca^{2+} homeostasis in conjunction with the presumed loss of reducing equivalents (NADPH/NADH) are both necessary and sufficient for β-lap-mediated apoptosis (Tagliarino *et al., J. Biol. Chem.,* Appendix).

AIM 2

The goal of aim 2 was to determine the role of calpain and its downstream targets in β-lap-induced apoptosis. This was done utilizing the NQO1-expressing MDA-468-NQ3 and MCF-7 cell lines.

Accomplishment of Stated Tasks for Aim 2:

We completed almost all tasks for specific aim 2. To determine calpain activation, we assayed proteolytic cleavage of the catalytic, as well as the regulatory, subunits of calpain in both NQO1-expressing MCF-7 cells and MDA-468-NQ3 cells using western blot analyses and specific antibodies that recognize the individual subunits (Task 1). The catalytic subunit of μ-calpain was cleaved in a temporal manner corresponding to substrate proteolysis (PARP, p53) and apoptosis (DNA fragmentation measured via the TUNEL assay) (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 1). In contrast, the other form of calpain that is ubiquitously expressed in mammalian cells, m-calpain, was not activated. These data further implicated μ-calpain as the sole calpain family member involved in β-lap-mediated apoptosis. To further

demonstrate calpain activation, casein zymogram activity assays will be performed (Task 1) that is more specific than the proposed flurogenic substrates. Also, in preliminary assays with the flurogenic substrates, positive control were not working, suggesting that the assay was either not working, or was not as sensitive as we needed to measure calpain activation.

To further demonstrate calpain activation, a calpastatin peptide containing the inhibitory region of calpastatin, and stable over-expression of full-length calpastatin in MCF-7 cells were used to inhibit calpain activity. The inhibitory peptide prevented PARP cleavage by menadione- and β-lap -treated cell extracts and purified μ-calpain in a dose-dependent manner in *in vitro* substrate cleavage assays (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 4). Stable over-expression of calpastatin in MCF-7 cells delayed apoptotic proteolysis and DNA fragmentation; calpastatin levels inversely correlated with apoptotic induction in NQO1-expressing breast cancer cells (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 1).

To conclusively demonstrate calpains role in β-lap-mediated apoptosis, we are collaborating with a group that made a calpain knock-out mouse. Currently, we are testing mouse embryonic fibroblasts that are deficient in both m- and μ-calpain activity (Task 1).

To determine μ-calpain and calpastatin localization after β-lap exposures, we utilized indirect immunofluorescence analyses in NQO1-expressing MCF-7 or MDA-MB-468 cells (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 3). After β-lap exposure, NQO1-expressing cells exhibited μ-calpain translocation to the nucleus in a temporal manner corresponding to autolysis of the regulatory and catalytic subunit of μ-calpain (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 3). Cells lacking NQO1 expression, MDA-468-Vec3 cells, or NQO1-expressing cells treated with dicumarol (an NQO1 inhibitor) did not undergo apoptosis at the doses used, and no translocation of μ-calpain was observed after β-lap exposures (data not

shown and Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 3). Furthermore, in NQO1-expressing cells treated with β-lap, calpastatin levels diminished concomitantly with μ-calpain activation, further implicating calpain activation (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 3). The nuclear membrane did not appear to be freely permeable to proteins, since NQO1 (a 30 kDa cytosolic enzyme) did not translocate to the nucleus at the time μ-calpain was activated (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 3). Translocation of μ-calpain to the nucleus after β-lap exposures was not unique to β-lap-induced apoptosis, but appeared to be unique to Ca^{2+}-mediated cell death pathways similar to β-lap exposed NQO1-expressing cells (e.g., menadione) (4,5), that also caused similar apparent activation and translocation to the nucleus of μ-calpain (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 3). Staurosporine, a protein kinase inhibitor and known activator of caspase-mediated apoptosis (23,24), did not elicit translocation of μ-calpain during apoptosis. However, ionomycin, a Ca^{2+} ionophore reported to activate calpains, exposure did result in similar μ-calpain translocation to those observed after β-lap or high dose menadione exposures (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 3). Calpastatin, the endogenous inhibitor of calpains, expression decreased and remained cytosolic when μ-calpain translocated to the nucleus, an event corresponding to μ-calpain activation, (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 3).

Another method to follow calpain translocation is to fuse calpain with the green fluorescence protein (GFP). Both N-terminal-GFP-μ-calpain and C-terminal-GFP-μ-calpain plasmids have been prepared and preliminary studies indicate that C-terminal-GFP-μ-calpain is not being expressed. Further work needs to be done to characterize and test these proteins in cells (Task 2); concerns still remain if the fused proteins will remain functional and if GFP will affect the translocation of calpain. However, these studies may not be necessary due to the

aforementioned experiments using indirect immunofluorescence (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 3).

Using *in vitro* transcription and translation, we were able to label proteins that are potential calpain substrates (e.g., PARP) with ^{35}S-methionine (^{35}S-met). ^{35}S-met-labelled-PARP was incubated with purified μ-calpain or MCF-7 cell extracts (untreated or β-lap-treated) for enzyme activation and substrate cleavage (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 4). μ-Calpain, as well as β-lap-treated or menadione-treated cell extracts, cleaved PARP in a similar manner, implicating μ-calpain was the activated protease required for atypical PARP cleavage in β-lap- and menadione- mediated apoptosis (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 4). Interestingly, addition of high levels of Ca^{2+} alone did not activate the proteolytic activity observed in β-lap-treated cell extracts; atypical PARP proteolysis was not enhanced in control extracts following high dose Ca^{2+}, compared to basal atypical PARP cleavage in untreated cell extracts. A calpastatin peptide containing the inhibitory region of calpastatin also prevented PARP cleavage by both drug-treated cell extracts and purified μ-calpain in a dose-dependent manner (Tagliarino *et al., J. Biol. Chem.,* Appendix II, Task 4).

These studies further elucidated a β-lap-induced apoptotic signal transduction pathway in NQO1-expressing breast cancer cells, as well as described a novel μ-calpain activation and translocation to the nucleus in apoptosis that may allow for potential improvements in breast cancer therapy.

Key Research Accomplishments

- Intracellular Ca^{2+} levels increased shortly after β-lap exposure of NQO1-expressing breast cancer cells (*J. Biol. Chem.*, Appendix).

- The increase in intracellular Ca^{2+} levels after β-lap exposure was prevented by pretreatment with dicumarol, an NQO1 specific inhibitor, and BAPTA-AM, an intracellular Ca^{2+} chelator (*J. Biol. Chem.*, Appendix).
- Mitochondrial membrane depolarization, ATP depletion, substrate proteolysis, DNA fragmentation, and cell death induced by β-lap in NQO1-expressing breast cancer cells was dependent upon increases in intracellular Ca^{2+} levels from the ER (*J. Biol. Chem.*, Appendix).
- Substrate proteolysis and DNA fragmentation were dependent upon Ca^{2+} from the extracellular milieu (*J. Biol. Chem.*, Appendix).
- After β-lap exposures in NQO1-expressing breast cancer cells, μ-calpain was activated and calpastatin levels (the endogenous inhibitor of calpains) were diminished in a manner corresponding to proteolytic cleavage events and DNA fragmentation (apoptosis) (Tagliarino *et al.*, *J. Biol. Chem.*, Appendix II).
- Purified μ-calpain, β-lap- and menadione- treated MCF-7 and MDA-468-NQ3 cell extracts exhibited the same proteolytic cleavage of PARP in *in vitro* assays (Tagliarino *et al.*, *J. Biol. Chem.*, Appendix II).
- A calpastatin inhibitory peptide blocked PARP cleavage by purified μ-calpain and β-lap-treated cell extracts in *in vitro* substrate assays (Tagliarino *et al.*, *J. Biol. Chem.*, Appendix II).
- Stable over-expression of calpastatin in MCF-7 cells, that possess low levels of endogenous calpastatin, delayed apoptotic proteolysis, DNA fragmentation and protected against cell death in clonogenic survival assays (Tagliarino *et al.*, *J. Biol. Chem.*, Appendix II).

- μ-Calpain translocation to the nucleus occurred in β-lap- and menadione- treated NQO1-expressing breast cancer cells at a time concomitant with substrate proteolysis and μ-calpain activation, dicumarol inhibited this translocation, and translocation was not observed in NQO1-deficient cells. Calpastatin levels decreased at times concomitant with μ-calpain activation and translocation (Tagliarino *et al., J. Biol. Chem.,* Appendix II).
- Calpain activation was independent of caspase activation; no known apoptotic caspases (3, 6, 7, 8, 9, 10, or 12) were activated in NQO1-expressing breast cancer cells exposed to β-lap (Tagliarino *et al., J. Biol. Chem.,* Appendix II).

Reportable Outcomes *(Manuscripts, Abstracts, presentations, patents, and licenses applied for and/or used):*

1. Manuscripts and Papers Published, In Press, and Submitted (See Appendix):

Tagliarino, C., Pink, J.J., Dubyak, G.R., Nieminen, A.L., and Boothman, D.A. Calcium is a Key Signaling Molecule in β-Lapachone-Mediated Cell Death. 2001 J. Biol. Chem. 276(22):19150-9.

Tagliarino, C., Pink, J.J., Wuerzberger-Davis, S.M., Simmers, S.M., and Boothman, D.A. μ-Calpain Activation in β-Lapachone-Mediated Apoptosis. 2001 J. Biol. Chem. *Submitted.*

S.M. Planchon, J.J. Pink, **C. Tagliarino,** W.G. Bornmann, M.E. Varnes, D.A. Boothman. "β-Lapachone-Induced Apoptosis in Human Prostate Cancer Cells: Involvement of NQO1/xip3." Exp. Cell Res. (2001) 267:95-106.

Miyamoto S, Huang TT, Wuerzberger-Davis S, Bornmann WG, Pink JJ, **Tagliarino C**, Kinsella TJ, Boothman DA. "Cellular and molecular responses to topoisomerase I poisons. Exploiting synergy for improved radiotherapy." Ann NY Acad Sci. 2000;922:274-92.

Pink, JJ, Wuerzberger-Davis, S., **Tagliarino, C.**, Planchon, SM, Yang, X, Froelich, CJ, Boothman, DA. "Activation of a Cysteine Protease in MCF-7 and T47D Breast Cancer Cells During β-Lapachone-Mediated Apoptosis." Exp. Cell Res. (2000) 255:144-155.

Pink, J.J., **Tagliarino, C.,** Planchon, S., Varnes, M., Simmers, S., and Boothman, D.A. Cell death pathways triggered by β-Lapachone. 2001; Free Radical Biology, *In press.*

2. Abstracts:

- Keystone Symposium Meeting on the Molecular Mechanisms of Apoptosis, Keystone, CO. "β-Lapachone Induces a Novel, Calpain-Like-Mediated Apoptotic Pathway." January 2001.
- American Association for Cancer Research Annual Meeting, New Orleans, LO. "Reactive Oxygen Species Are Not the Mediators of β-Lapachone-Induced Apoptosis in Breast Cancer Cells." March 2001.
- Case Western Reserve University Graduate Student Symposium, "Exploiting an NQO1-Directed, Calpain-Like-Mediated Apoptotic Pathway for Breast Cancer Therapy." May 2000.

3. Presentations:

Invited Speaker, "β-Lapachone, A Novel Chemotherapeutic Agent." John Carroll University, Dr. Jim Lissemore, host; Student Seminar Speaker. September 2000.

4. **Patents, licenses, development of cell lines or funding applied for:** None
5. **Degrees Obtained**: Colleen Tagliarino will obtain her Ph.D. from Case Western Reserve University in January 2002.

Conclusions

The research ongoing and proposed in this proposal addresses a novel apoptotic agent, β-lapachone, shown to be active agent human breast and prostate cancer cells. Cell death mediated by this compound is dependent upon alterations in intracellular Ca^{2+} homeostasis that mediates changes in mitochondrial membrane potential, ATP depletion, specific and unique substrate proteolysis, and DNA fragmentation. β-Lap also appears to work through a novel, noncaspase-mediated, apoptotic pathway with similarities to μ-calpain-mediated apoptosis. Since many cancers arise due to alterations in p53, pRb and caspases, and β-lap induces apoptosis independent of p53 or pRb status, as well as caspase activation, development of this compound for breast cancer therapy could greatly improve therapy of these diseases.

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Appendix Items

Tagliarino, C., Pink, J.J., Dubyak, G.R., Nieminen, A.L., and Boothman, D.A. Calcium is a Key Signaling Molecule in β-Lapachone-Mediated Cell Death. 2001 J. Biol. Chem. 276(22):19150-9.

Tagliarino, C., Pink, J.J., Wuerzberger-Davis, S.M., Simmers, S.M., and Boothman, D.A. μ-Calpain Activation in β-Lapachone-Mediated Apoptosis. 2001 J. Biol. Chem. *Submitted.*

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Calcium Is a Key Signaling Molecule in β-Lapachone-mediated Cell Death*

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β-Lapachone (β-Lap) triggers apoptosis in a number of human breast and prostate cancer cell lines through a unique apoptotic pathway that is dependent upon NQO1, a two-electron reductase. Downstream signaling pathway(s) that initiate apoptosis following treatment with β-Lap have not been elucidated. Since calpain activation was suspected in β-Lap-mediated apoptosis, we examined alterations in Ca^{2+} homeostasis using NQO1-expressing MCF-7 cells. β-Lap-exposed MCF-7 cells exhibited an early increase in intracellular cytosolic Ca^{2+}, from endoplasmic reticulum Ca^{2+} stores, comparable to thapsigargin exposures. 1,2-Bis-(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid-acetoxymethyl ester, an intracellular Ca^{2+} chelator, blocked early increases in Ca^{2+} levels and inhibited β-Lap-mediated mitochondrial membrane depolarization, intracellular ATP depletion, specific and unique substrate proteolysis, and apoptosis. The extracellular Ca^{2+} chelator, EGTA, inhibited later apoptotic end points (observed >8 h, *e.g.* substrate proteolysis and DNA fragmentation), suggesting that later execution events were triggered by Ca^{2+} influxes from the extracellular milieu. Collectively, these data suggest a critical, but not sole, role for Ca^{2+} in the NQO1-dependent cell death pathway initiated by β-Lap. Use of β-Lap to trigger an apparently novel, calpain-like-mediated apoptotic cell death could be useful for breast and prostate cancer therapy.

β-Lap[1] is a naturally occurring compound present in the bark of the South American Lapacho tree. It has antitumor activity against a variety of human cancers, including colon, prostrate, promyelocytic leukemia, and breast (1–3). β-Lap was an effective agent (alone and in combination with taxol) against human ovarian and prostate xenografts in mice, with little host toxicity (4). We recently demonstrated that β-Lap kills human breast and prostate cancer cells by apoptosis, a cytotoxic response significantly enhanced by NAD(P)H:quinone oxidoreductase (NQO1, E.C. 1.6.99.2) enzymatic activity (5).[2] β-Lap cytotoxicity was prevented by co-treatment with dicumarol (an NQO1 inhibitor) in NQO1-expressing breast and prostate cancer cells (5).[2] NQO1 is a cytosolic enzyme elevated in breast cancers (6) that catalyzes a two-electron reduction of quinones (*e.g.* β-Lap, menadione), utilizing either NADH or NADPH as electron donors. Reduction of β-Lap by NQO1 presumably leads to a futile cycling of the compound, wherein the quinone and hydroquinone form a redox cycle with a net concomitant loss of reduced NAD(P)H (5).

Apoptosis is an evolutionarily conserved pathway of biochemical and molecular events that underlie cell death processes involving the stimulation of intracellular zymogens. The process is a genetically programmed form of cell death involved in development, normal turnover of cells, and in cytotoxic responses to cellular insults. Once apoptosis is initiated, biochemical and morphological changes occur in the cell. These changes include: DNA fragmentation, chromatin condensation, cytoplasmic membrane blebbing, cleavage of apoptotic substrates (*e.g.* PARP, lamin B), and loss of mitochondrial membrane potential with concomitant release of cytochrome *c* into the cytoplasm (7–9). Apoptosis is a highly regulated, active process that requires the participation of endogenous cellular enzymes that systematically dismantle the cell. The most well characterized proteases in apoptosis are caspases, aspartate-specific cysteine proteases, that work through a cascade that can be initiated by mitochondrial membrane depolarization leading to the release of cytochrome *c* and Apaf-1 into the cytoplasm (10), that then activates caspase 9 (11). Non-caspase-mediated pathways are less understood.

We previously showed that apoptosis following β-Lap administration was unique, in that an ~60-kDa PARP cleavage fragment, as well as distinct intracellular proteolytic cleavage of p53, were observed in NQO1-expressing breast or prostate cancer cells (5).[2] These cleavage events were distinct from those observed when caspases were activated by topoisomerase I poisons, staurosporine, or administration of granzyme B (5, 12, 13). Furthermore, β-Lap-mediated cleavage events were blocked by administration of global cysteine protease inhibitors, as well as extracellular Ca^{2+} chelators (12). Based on these data, we concluded that β-Lap exposure of NQO1-expressing breast and prostate cancer cells caused the activation of a Ca^{2+}-dependent protease with properties similar to calpain; in particular, the p53 cleavage pattern of β-Lap-exposed

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[1] The abbreviations used are: β-Lap, β-lapachone; MCP, MCF-7:WS8; NQO1, NAD(P)H:quinone oxidoreductase, DT-diaphorase (E.C. 1.6.99.2); PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; ER, endoplasmic reticulum; TG, thapsigargin; STS, staurosporine; BAPTA-AM, 1,2-bis-(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid-acetoxymethyl ester.

[2] S. M. Planchon, C. Tagliarino, J. J. Pink, W. G. Bornmann, M. E. Varnes, and D. A. Boothman. *Exp. Cell Res.*, in press.

cells was remarkably similar to the pattern observed after calpain activation (14, 15).

Ca^{2+} is recognized as an important regulator of apoptosis (16–21). The cytoplasmic Ca^{2+} concentration is maintained at ~100 nM in resting cells by relatively impermeable cell membranes, active extrusion of Ca^{2+} from the cell by plasma membrane Ca^{2+}-ATPases, plasma membrane Na^+/Ca^{2+} exchangers, and active uptake of cytosolic Ca^{2+} into the endoplasmic reticulum (ER) by distinct Ca^{2+}-ATPases. In contrast, the concentration of Ca^{2+} in the extracellular milieu and in the ER is much higher (in the millimolar range). Evidence for involvement of Ca^{2+} influx into the cytosol as a triggering event for apoptosis has come from studies with specific Ca^{2+} channel blockers that abrogate apoptosis in regressing prostate following testosterone withdrawal (22). Other support for the involvement of Ca^{2+} in apoptosis comes from the observation that agents that directly mobilize Ca^{2+} (*e.g.* Ca^{2+} ionophores or the sarcoplasmic reticulum Ca^{2+}-ATPase pump inhibitor, thapsigargin, TG) can trigger apoptosis in diverse cell types (23–27). Inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase pump by TG causes a transient increase in cytoplasmic Ca^{2+} from ER Ca^{2+} stores, and a later influx of Ca^{2+} from the extracellular milieu, leading to the induction of apoptotic cell death (24, 27, 28). Consequently, emptying of intracellular Ca^{2+} stores may trigger apoptosis by disrupting the intracellular architecture and allowing key elements of the effector machinery (*e.g.* Apaf-1) to gain access to their substrates (*e.g.* caspase 9). Ca^{2+} has also been shown to be necessary for apoptotic endonuclease activation, eliciting DNA cleavage after many cellular insults (29–31). Buffering intracellular Ca^{2+} released from stored Ca^{2+} pools (*e.g.* ER) with BAPTA-AM, or removal of extracellular Ca^{2+} with EGTA, can protect cells against apoptosis (32, 33). Therefore, increases in intracellular Ca^{2+} levels appear to be important cell death signals in human cancer cells that might be exploited for anti-tumor therapy. Finally, Ca^{2+} may act as a signal for apoptosis by directly activating key proapoptotic enzymes (*e.g.* calpain); however, these proteolytic responses are poorly understood. The role of Ca^{2+} in cell death processes involving caspase activation has been examined in detail (28, 34–36). However, the role of Ca^{2+} in non-caspase-dependent cell death responses is relatively unexplored.

Recent studies have suggested that alterations in mitochondrial homeostasis play an essential role in apoptotic signal transduction induced by cytotoxic agents (37, 38). Various apoptotic stimuli have been shown to induce mitochondrial changes, resulting in release of apoptogenic factors, apoptosis-inducing factor (39), and mitochondrial cytochrome *c* (9) into the cytoplasm. These changes are observed during the early phases of apoptosis in human epithelial cells, and were linked to the initial cascade of events, sending the cell to an irreversible suicide pathway. During high, sustained levels of cytosolic Ca^{2+}, mitochondrial Ca^{2+} uptake is driven by mitochondrial membrane potential to maintain Ca^{2+} homeostasis in the cytosol. In de-energized mitochondria, Ca^{2+} can be released by a reversal of this uptake pathway (40). These data, therefore, linked changes in Ca^{2+} homeostasis and mitochondrial membrane potential to the initiation of apoptosis. Li *et al.* (41) reported that β-Lap caused a decrease in mitochondrial membrane potential with release of cytochrome *c* into the cytoplasm in a number of human carcinoma cell lines, shortly after drug addition. Other alterations in metabolism (*e.g.* ATP depletion) have not been examined in β-Lap-treated cells.

We previously characterized the activation of a novel cysteine protease in various breast cancer cell lines with properties similar to the Ca^{2+}-dependent cysteine protease, calpain, after exposure to β-Lap (12). Using NQO1-expressing breast cancer cells, we show that β-Lap elicits a rise in intracellular Ca^{2+} levels shortly after drug administration that eventually leads to apoptosis. This paper suggests a critical, but not sufficient, role for Ca^{2+} in the cell death pathway initiated by NQO1-dependent bioactivation of β-Lap. Possible combinatorial effects (*e.g.* NAD(P)H depletion as well as intracellular calcium alterations) that initiate β-Lap-mediated apoptosis in NQO1-expressing breast cancer cells will be discussed.

EXPERIMENTAL PROCEDURES

Reagents—β-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2b]pyran-5,6-dione) was synthesized by Dr. William G. Bornmann (Memorial Sloan Kettering, New York), dissolved in dimethyl sulfoxide at 10 mM, and the concentration verified by spectrophotometric analysis (2, 5). EGTA, Hoescht 33258, and thapsigargin were obtained from Sigma. BAPTA-AM (1,2-bis-(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetra-(acetoxymethyl ester)) was obtained from Calbiochem (La Jolla, CA). JC-1 (5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) and Fluo-4-AM were obtained from Molecular Probes, Inc. (Eugene, OR).

Cell Culture—MCF-7:WS8 (MCF-7) human breast cancer cells were obtained from Dr. V. Craig Jordan, (Northwestern University, Chicago, IL). MDA-MB-468 cells were obtained from the American Type Culture Collection and transfected with NQO1 cDNA in the pcDNA3 constitutive expression vector as described previously (5). Tissue culture components were purchased from Life Technologies, Inc., unless otherwise stated. MCF-7 cells were grown in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum, in a 37 °C humidified incubator with 5% CO_2, 95% air atmosphere as previously described (2, 5). For all experiments, log-phase breast cancer cells were exposed to 5 μM β-Lap for 4 h (unless otherwise indicated), after which fresh medium was added and cells were harvested at various times post-treatment.

TUNEL Assay—Cells were seeded at 1×10^6 cells/10-cm Petri dish and allowed to grow for 24 h. Log-phase cells were then pretreated for 30 min with 10 μM BAPTA-AM, 3 mM EGTA, or 50 μM dicumarol followed by a 4-h pulse of 5 μM β-Lap, as described above, or 24 h treatment of 10 μM ionomycin or 1 μM staurosporine. Medium was collected from experimental as well as control conditions 24 h later, and attached along with floating cells were monitored for apoptosis using TUNEL 3′-biotinylated DNA end labeling via the APO-DIRECT kit (Pharmingen, San Diego, CA) as described (5). Apoptotic cells were analyzed and quantified using an EPICS XL-MCL flow cytometer that contained an air-cooled argon laser at 488 nm, 15 mW (Beckman Coulter Electronics; Miami, Fl), and XL-MCL acquisition software provided with the instrument.

Cell Growth Assays—MCF-7 cells were seeded at 5×10^4 cells per well in a 12-well plate and allowed to attach overnight. The following day, log-phase cells were pretreated for 30 min with 5 μM BAPTA-AM, followed by a 4-h pulse of β-Lap (0–5 μM). Drugs were removed and fresh medium added. Cells were allowed to grow for an additional 6 days. DNA content (a measure of cell growth) was determined by fluorescence using Hoechst dye 33258 as described (5) and changes in growth were monitored using a PerkinElmer HTS 7000 Plus Bio Assay Plate Reader (Norwalk, CT) with 360 and 465 nm excitation and emission filters, respectively. Data were expressed as relative growth, T/C (treated/control), using experiments performed at least twice.

Confocal Microscopy—MCF-7 cells were seeded at $2–3 \times 10^5$ cells per 35-mm glass bottom Petri dishes (MatTek Corp., Ashland, MA) and allowed to attach overnight. Cells were rinsed twice in a Ca^{2+}/Mg^{2+} balanced salt solution (BSS, 130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM HEPES, pH 7.5, 5 mM glucose, 1 mg/ml bovine serum albumin) and loaded with the Ca^{2+}-sensitive fluorescent indicator, fluo-4-AM (5 μM), in BSS for ~20–30 min at 37 °C. Cells were rinsed twice in BSS and incubated for an additional 20 min at 37 °C to allow for hydrolysis of the AM-ester. Cells were imaged with a Zeiss 410 confocal microscope (Thornwood, NY) equipped with a ×63 N.A. 1.4 oil immersion planapochromat objective at room temperature (the same results were observed at room temperature and 37 °C). Confocal images of fluo-4 fluorescence were collected using a 488-nm excitation light from an argon/krypton laser, a 560-nm dichroic mirror, and a 500–550 nm band-pass barrier filter. Three basal images were collected before drug addition (8 μM β-Lap, ± 50 μM dicumarol or 200 nM TG). The mean pixel intensity was set to equal one for analyses of fold-increase in fluo-4 fluorescence intensity. Subsequently, images were collected after the indicated treatments at 90-s intervals. BAPTA-AM (20 μM) was co-

loaded with fluo-4-AM where indicated. Mean pixels were determined in regions of interest for individual cells at each time point.

Mitochondrial Membrane Potential Determinations—MCF-7 cells were seeded at 2.5–3 × 10^5 cells per 6-well plate, and allowed to grow for 24 h. Log-phase cells were pretreated for 30 min with 10 μM BAPTA-AM, 3 mM EGTA, or 50 μM dicumarol followed by a 4-h pulse of 5 μM β-Lap, unless otherwise indicated. Cells were trypsinized and resuspended in phenol red-minus RPMI medium for analyses. Cells were maintained at 37 °C for the duration of the experiment, including during analyses. Prior to analyses, cells were loaded with 10 μg/ml JC-1 for 9–14 min and samples were analyzed using a Beckman Coulter EPICS Elite ESP (Miami, FL) flow cytometer. JC-1 monomer and aggregate emissions were excited at 488 nM and quantified using Elite acquisition software after signal collection through 525- and 590-nm band pass filters, respectfully. Shifts in emission spectra were plotted on bivariant dot plots, on a cell-by-cell basis, to determine relative mitochondrial membrane potential of treated and control cells.

ATP Measurements—Cells were seeded at 2.5 × 10^5 cells per well in 6-well dishes and allowed to attach for 24 h. Fresh medium was added to the cells along with Ca^{2+} chelators or dicumarol 30 min prior to β-Lap exposure (4 h unless otherwise indicated). Floating cells were collected, pelleted, and lysed in 1.67 M perchloric acid. Attached cells were lysed directly in 1.67 M perchloric acid. Following a 20-min incubation at room temperature, attached cells were scraped and transferred to corresponding microcentrifuge tube, cooled on ice for several minutes, and spun to pellet protein precipitates. Deproteinized samples were neutralized with 3.5 M KOH and HEPES/KOH (25 mM HEPES, 15 mM KOH, pH 8), and incubated on ice for 15 min. Precipitates were removed by centrifugation and samples stored at −20 °C. Cell extracts were analyzed for ATP and ADP levels using a luciferase-based bioluminescent assay and rephosphorylation protocols, as described (42).

Western Blot Analyses—Whole cell extracts from control or β-Lap-exposed MCF-7 cells were prepared and analyzed by SDS-polyacrylamide gel electrophoresis/Western blot analyses as previously described (2, 5, 12). Loading equivalence and transfer efficiency were monitored by Western blot analyses of proteins that are known to be unaltered by experimental treatments (2), and using Ponceau S staining of the membrane, respectively. Probed membranes were then exposed to x-ray film for an appropriate time and developed. Dilutions of 1:10,000 for the C-2–10 anti-PARP antibody (Enzyme Systems Products, Livermore, CA), and 1:2000 for anti-p53 DO-1 and anti-lamin B (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used as described (2, 12).

RESULTS

Ca^{2+} Chelators Prevent β-Lap-induced Apoptotic DNA Fragmentation and Protect against Cell Death—Log-phase MCF-7 cells were treated for 4 h with 5 μM β-Lap, fresh medium was then applied, and cells were harvested 24 h later and analyzed for DNA fragmentation (*i.e.* apoptotic cells staining positive in a TUNEL assay). Treatment of MCF-7 cells with β-Lap resulted in >90% apoptotic cells (Fig. 1, *A* and *B*). However, MCF-7 cells exposed to a 30-min pretreatment with 10 μM BAPTA-AM or 3 mM EGTA, followed by a 4-h pulse of 5 μM β-Lap, exhibited only 20 or 39% apoptotic cells, respectively, in 24 h.

To examine whether BAPTA-AM could affect β-Lap lethality, we measured relative growth of MCF-7 cells with or without exposure to β-Lap, and in the presence or absence of BAPTA-AM. MCF-7 cells were treated for 30 min with 5 μM BAPTA-AM, subsequently exposed to a 4-h pulse of β-Lap (1.5−5 μM), and relative cell growth was measured 6 days later (Fig. 1*C*). The LD_{50} dose of β-Lap in MCF-7 cells was ~2.5 μM in colony forming assays, which correlated well with IC_{50} relative growth inhibition, as measured by DNA content (2, 5). At 1.5 μM β-Lap, cells exhibited little or no toxicity. At β-Lap doses of 3 or 5 μM, cells exhibited considerable toxicity, >90% growth inhibition, as previously reported (2, 5). Toxicity was significantly prevented by 5 μM BAPTA-AM pretreatment. BAPTA-AM pretreated cells exhibit only 44 and 73% growth inhibition after 3 or 5 μM β-Lap treatments, respectively (Fig. 1*C*). BAPTA alone did not affect MCF-7 cell growth compared with untreated controls.

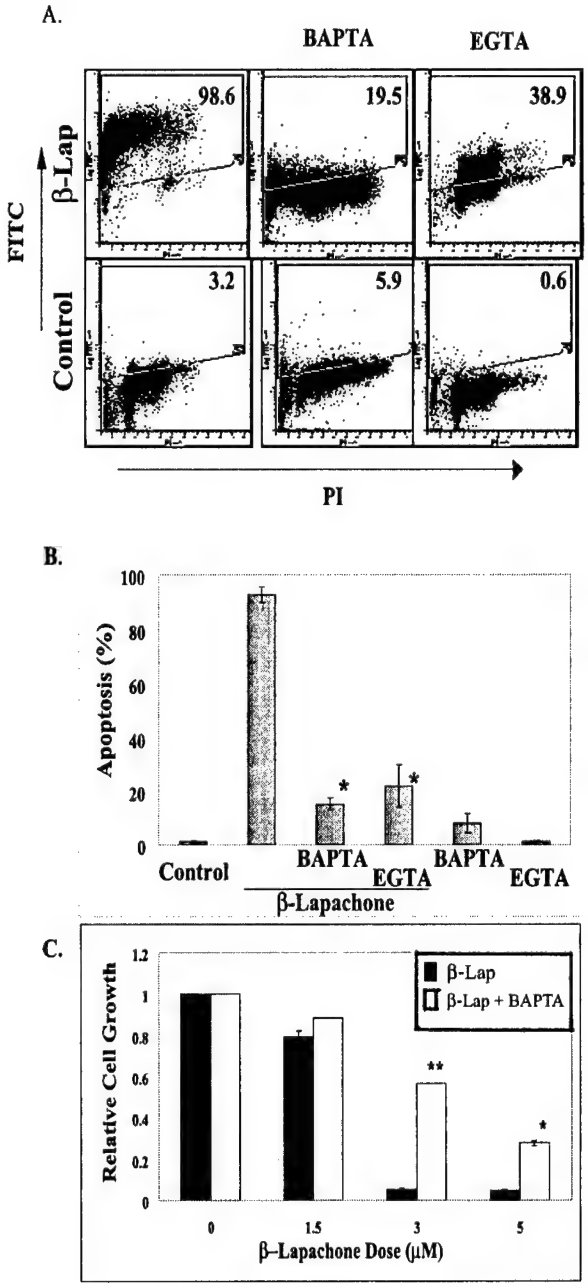

FIG. 1. **β-Lap-mediated apoptosis and relative cell growth is Ca^{2+}-dependent.** DNA fragmentation was assessed using the TUNEL assay. Log phase MCF-7 cells were treated with the indicated Ca^{2+} chelator for 30 min prior to a 4-h pulse of 5 μM β-Lap. TUNEL assays were performed to monitor apoptosis 24 h after β-Lap addition (*A* and *B*). *A*, shown are the results of any one experiment from studies performed at least three times. The *number* in the *upper right* corner represents percent cells staining positive in the TUNEL assay. Results are graphically summarized in *B* as the average of at three independent experiments, mean ± S.E. Student's *t* test for paired samples, experimental group compared with MCF-7 cells treated with β-Lap alone are indicated (* $p < 0.01$). *C*, cells were exposed to a 4-h pulse of various concentrations of β-Lap either alone (*closed*), or after a 30-min pretreatment with 5 μM BAPTA-AM (*open*). Relative DNA per well was determined by Hoescht 33258 fluorescence, and graphed as relative growth (treated/control DNA); mean relative DNA per well, ± S.E. Shown are representative results of experiments performed at least twice. Student's *t* test for paired samples, experimental group compared with MCF-7 cells treated with β-Lap alone are indicated (*, $p < 0.05$; and **, $p < 0.005$).

Ca^{2+} Chelators Do Not Block Apoptosis Induced by Other Agents—It was possible based on the data in Fig. 1 that calcium chelators may block β-Lap-mediated apoptosis by sequestering

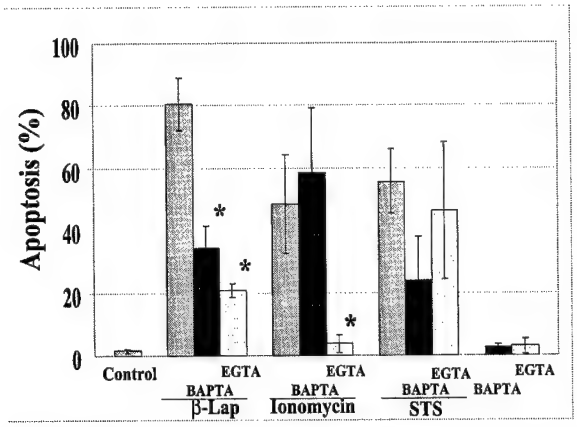

FIG. 2. **Ca^{2+} chelators did not block Ca^{2+}-activated endonuclease activation after β-Lap.** NQO1-expressing MDA-468-NQ3 cells (generated from non-expressing human breast cancer cells (5)) were treated with either 3 mM EGTA or 30 μM BAPTA-AM for 30 min prior to drug addition; either a 4-h pulse of 8 μM β-Lap, or 24 h continuous treatment of 10 μM ionomycin or 1 μM STS. Cells were then analyzed using the TUNEL assay for DNA fragmentation. Shown are mean ± S.E. of at least two independent experiments. Student's *t* test for paired samples, experimental group compared with cells treated with drug alone are indicated (*, $p < 0.05$).

calcium required for the activation of apoptotic endonucleases. We, therefore, examined both intra- and extracellular Ca^{2+} chelators for their ability to prevent apoptosis in NQO1-transfected MDA-468 (MDA-468-NQ3) cells induced by β-Lap, ionomycin (which induces Ca^{2+}-mediated cell death (36)), and staurosporine (STS, which inhibits protein kinase C and works via a caspase-mediated cell death pathway (43, 44)). We used MDA-468-NQ3 cells to assay for caspase-mediated endonuclease activation and DNA fragmentation since they express the endonuclease-activating caspase 3, unlike MCF-7 cells (45). We previously demonstrated that MDA-468-NQ3 cells responded similarly to β-Lap as MCF-7 cells (Fig. 2 and Ref. 5). EGTA significantly protected MDA-468-NQ3 cells against ionomycin-induced apoptosis, but not against STS-induced apoptosis (Fig. 2). MDA-468-NQ3 cells treated for 24 h with 10 μM ionomycin exhibited 49% apoptotic cells, whereas, MDA-468-NQ3 cells pretreated for 30 min with 3 mM EGTA followed by a 24-h exposure to ionomycin exhibited only 4% apoptotic cells. Cells treated for 24 h with 1 μM STS in the absence or presence of 3 mM EGTA exhibited 56 and 46% apoptosis, respectively. BAPTA-AM (10 μM) did not significantly block apoptosis induced by ionomycin. BAPTA-AM pretreatment of STS-exposed MDA-468-NQ3 cells did not significantly decrease apoptosis ($p < 0.4$) compared with cells exposed to STS alone; the modest effect of BAPTA-AM on STS-induced apoptosis may reflect the Ca^{2+} dependence of the apoptotic endonucleases involved in this response. Neither BAPTA-AM nor EGTA alone elicited apoptotic responses at the doses used in the aforementioned experiments (Figs. 1*B* and 2). Furthermore, preliminary data suggest that DFF45 (ICAD) was cleaved in NQO1-expressing MCF-7 or MDA-468-NQ3 cells at 8 h after β-Lap treatment, in a temporal manner corresponding to the induction of apoptosis (data not shown). Cleavage of DFF45, an endogenous inhibitor of the magnesium-dependent and Ca^{2+}-independent apoptotic endonuclease, DFF40 (CAD), suggests that DFF40 is activated following treatment with β-Lap. Taken together with results in Fig. 1, these data strongly suggest that a rise in intracellular Ca^{2+} levels is part of a critical signaling pathway for the induction of apoptosis in NQO1-expressing human breast cancer cells following β-Lap exposure.

Exposure of NQO1-expressing MCF-7 Cells to β-Lap Results in Increased Intracellular Ca^{2+}—We next directly examined whether intracellular Ca^{2+} levels were increased in log-phase MCF-7 cells after β-Lap treatment using the cell-permeant intracellular Ca^{2+} indicator dye, fluo-4. Cells were loaded with 5 μM fluo-4-AM, and where indicated, 20 μM BAPTA-AM, incubated for ~25 min to allow for the dye to permeate cells, rinsed, and then incubated for an additional ~20 min for hydrolysis of the AM-ester. Following drug addition, images were collected every 90 s for ~60 min using confocal microscopy. Three basal images were recorded before drug addition and average pixels per cell were determined (indicative of fluo-4 fluorescence and, therefore, basal intracellular Ca^{2+} levels) and used for analyses over time. The fluorescence of basal images were averaged and set to equal one; fold increases were determined from changes in fluo-4 fluorescence over control.

After exposure to 8 μM β-Lap, MCF-7 cells exhibited an ~2-fold increase in fluo-4 fluorescence from 4 to 9 min, after which time Ca^{2+} levels returned to basal levels in a majority of cells examined (43 of 50, 86%) (Fig. 3*A*). The rise in intracellular Ca^{2+} levels in MCF-7 cells following β-Lap exposure was prevented by preloading cells with BAPTA-AM (20 μM) (Fig. 3*B*). Interestingly, not all β-Lap-exposed MCF-7 cells were affected by pretreatment with BAPTA-AM; 3 of 26 cells (12%) exhibited a rise in intracellular Ca^{2+} levels after exposure to β-Lap despite the presence of this Ca^{2+} chelator. However, BAPTA-AM pretreated MCF-7 cells that did exhibit a rise in intracellular Ca^{2+} levels following β-Lap treatment exhibited a similar, but delayed Ca^{2+} increase (10–20 min), as compared with β-Lap-exposed MCF-7 cells in the absence of BAPTA-AM (4–9 min). This may be due to a saturation of the chelator or heterogeneity of the tumor cell population. These results are consistent with previous reports that the buffering capacity of BAPTA-AM may be overwhelmed with time (34, 46). Higher doses of BAPTA-AM were not used due to toxicity caused by the drug alone (data not shown).

Since the ER is a major store of Ca^{2+} in the cell, we tested if the initial rise in intracellular Ca^{2+} levels after exposure of MCF-7 cells to β-Lap was due to release of Ca^{2+} from this organelle. If β-Lap exposure led to release of Ca^{2+} stored in the ER, then TG (a sarcoplasmic reticulum Ca^{2+}-ATPase pump inhibitor) administration should not cause additional Ca^{2+} release. Similarly, if the sequence of drug administration were reversed, additional Ca^{2+} release would also not be observed. When β-Lap was added after TG-induced depletion of ER Ca^{2+} stores, no measurable rise in intracellular Ca^{2+} levels occurred in 25 of 27 (93%) cells analyzed (Fig. 3*C*). Similarly, when TG was added to cells after β-Lap, only 1 of 18 (6%) cells that initially responded to β-Lap exhibited a rise in intracellular Ca^{2+} levels following subsequent TG administration (Fig. 3*D*). At the end of the experiment, all cells analyzed remained responsive to ionomycin. Thus, cells exposed to β-Lap and/or TG were still capable of altering Ca^{2+} levels, and the Ca^{2+} indicator dye was not saturated. We noted that the increase in fluo-4 fluorescence (2–3-fold over basal levels, Fig. 3*A*) in MCF-7 cells observed after exposure to β-Lap was comparable to that elicited by TG (1.5–2.5-fold over basal levels, Fig. 3*C*), further suggesting that the two agents mobilized the same ER pool of Ca^{2+}. All cells analyzed started with comparable basal levels of Ca^{2+} and appeared to load equal amounts of the indicator dye, as determined by basal fluorescence (measured by pixels per cell) at the beginning of each analysis; relative basal fluo-4 fluorescence for each experiment in Fig. 3 were: A, 56 ± 7; B, 52 ± 7; C, 78 ± 8; D, 79 ± 8 S.E. Untreated or BAPTA-AM-loaded MCF-7 cells did not show any fluctuations in basal Ca^{2+} levels during the time course of the experiment, nor did any of the drugs interfere with the Ca^{2+} indicator dye (data not shown).

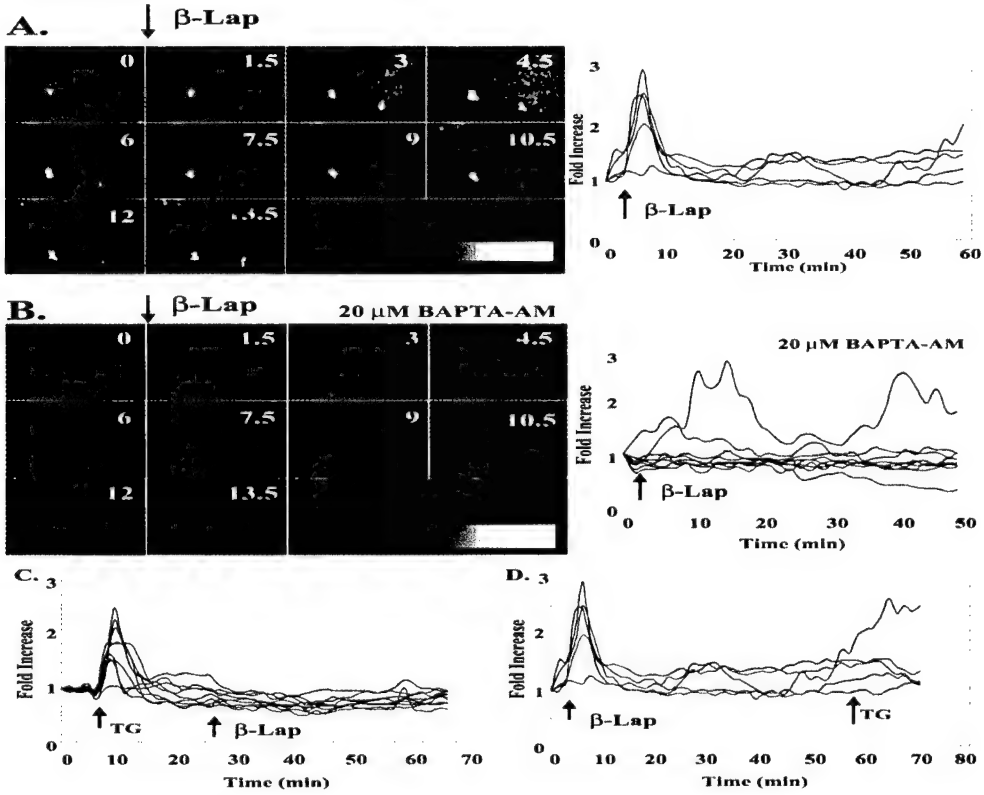

FIG. 3. **Intracellular Ca^{2+} changes after β-Lap.** Intracellular Ca^{2+} levels were measured in live cells via confocal microscopy using the Ca^{2+} indicator dye, fluo-4-AM. MCF-7 cells were loaded with either fluo-4-AM alone (*A*, *C*, and *D*) or fluo-4-AM and 20 μM BAPTA-AM (*B*). β-Lap (8 μM) was added to cells after basal images were recorded. Images were collected every 90 s for 45–75 min, as indicated. The *number* in the *upper right corner* of each Ca^{2+} image represents the time (min) after β-Lap addition. *A*, representative cells before and after β-Lap treatments are shown as pseudocolored images. These results are also displayed in *graph form* showing fold change (as compared with basal levels) in fluo-4 fluorescence in cells after β-Lap treatment over time, with or without co-loading of BAPTA-AM (*A* and *B*). *C*, TG (200 nM) was added to MCF-7 cells after basal images were recorded. Once fluo-4 fluorescence returned to basal levels, cells were subsequently exposed to β-Lap. *D*, β-Lap was added to MCF-7 cells after basal images were recorded. After fluo-4 fluorescence returned to basal levels, TG was subsequently added to the cells. Each *line* represents the change in fluo-4 fluorescent emission of an individual cell over time; each graph is representative of one of at least three independent experiments.

Loss of Mitochondrial Membrane Potential After β-Lap Is Attenuated by Intracellular, but Not Extracellular, Ca^{2+} Chelation—Mitochondrial membrane potential was previously shown to drop from a hyperpolarized state to a depolarized state after treatment of various human cancer cells with β-Lap (41). A drop in mitochondrial membrane potential in β-Lap-treated cells was accompanied by a concomitant release of cytochrome *c* into the cytosol (41). To explore whether early changes in intracellular Ca^{2+} levels were upstream of mitochondrial changes in NQO1-expressing breast cancer cells, log phase MCF-7 cells were pretreated for 30 min with either 10 μM BAPTA-AM or 3 mM EGTA and then exposed to 5 μM β-Lap for 4 h. Prior to analyses, cells were loaded with JC-1, a cationic dye commonly used to monitor alterations in mitochondrial membrane potential (47, 48). Mitochondrial depolarization measurements using JC-1 were indicated by a decrease in the red/green fluorescence intensity ratio (a movement of events from *upper left* to *lower right*, Fig. 4), as seen following a 10-min treatment with the potassium ionophore, valinomycin (100 nM), which causes a collapse of mitochondrial membrane potential by uncoupling mitochondrial respiration (Fig. 4*e*) (49); cells in the *upper left-hand* quadrant exhibited high mitochondrial membrane potential, whereas, cells in the *lower right-hand* quadrant have low mitochondrial membrane potential and are depolarized. Cells in the *upper right-hand* quadrant exhibited intermediate membrane potential. Mitochondrial membrane potential decreased in MCF-7 cells in a time- and dose-dependent manner following exposure to β-Lap (Figs. 4, *a-d*, and data not shown). By 4 h, the majority of β-Lap-treated MCF-7 cells exhibited low mitochondrial membrane potential (53%), while the majority of control cells maintained high mitochondrial membrane potential (51%) (Fig. 4, *b, a* and *g, f*, respectively). This drop in mitochondrial membrane potential observed 4 h after treatment with β-Lap (low, 53%) was abrogated by pretreatment with BAPTA-AM (low, 23%), but not by EGTA (low, 48%) (Fig. 4, *g-i*, respectively). Pretreatment with 10 μM BAPTA-AM prevented the decrease in mitochondrial membrane potential (low, 23%); however, BAPTA-AM did not maintain β-Lap-exposed cells in a high-potential state (high, 28%) as observed in control untreated cells (high, 51%). Approximately half of the BAPTA-AM-exposed cells were in an intermediate membrane potential state (45%) (Fig. 4*h*). We noted, however, that BAPTA-AM or EGTA exposures alone caused depolarization of the mitochondria, with a majority of the cells residing in the same intermediate energized state as observed following BAPTA-AM and β-Lap (Fig. 4, *j-k*). Therefore, BAPTA-AM prevented mitochondrial depolarization induced by β-Lap to the same extent as in cells treated with BAPTA-AM alone. Pretreatment with 3 mM EGTA did not affect the loss of mitochondrial membrane potential caused by β-Lap (low 48%), implying that an early rise in intracellular Ca^{2+} levels from intracellular stores was sufficient to cause a drop in mitochondrial membrane potential, and that extracellular calcium was not needed for these effects in β-Lap-treated cells (Fig. 4, *h-i*).

Loss of ATP After β-Lap Is Attenuated by Intracellular Ca^{2+} Chelation—The bioactivation of β-Lap by NQO1 is thought to lead to a futile cycling between quinone and hydroquinone forms of the compound, presumably due to the instability of the

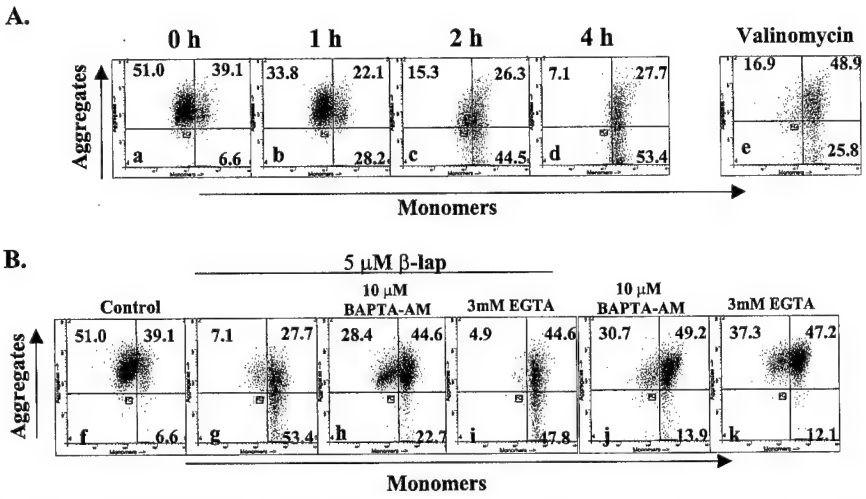

FIG. 4. **β-Lap-induced loss of mitochondrial membrane potential is mediated by alterations in Ca^{2+} homeostasis.** Mitochondrial membrane potential was measured in control or drug-treated MCF-7 cells with the JC-1 dye. *A*, cells were treated with 5 μM β-Lap and assayed for changes in mitochondrial membrane potential at 1, 2, and 4 h post-treatment. Exposure of MCF-7 cells to 100 nM valinomycin for 15 min served as a positive control as described (49). Cells in the *upper left-hand* quadrant exhibit high mitochondrial membrane potential, while cells in the *lower right-hand* quadrant exhibit low mitochondrial membrane potential. *B*, cells were treated for 30 min with either 10 μM BAPTA-AM or 3 mM EGTA prior to a 4-h treatment with 5 μM β-Lap. At 4 h, cells were harvested for analyses of changes in mitochondrial membrane potential using JC-1 as described above. Shown are representative experiments performed at least three times, and *numbers* in each quadrant represent the average of cells in that quadrant of at least three independent experiments. S.E. for any single number was not more than 11%.

hydroquinone form of β-Lap (5). This futile cycling led to depletion of NADH and NADPH, electron donors for NQO1 in *in vitro* assays (5). Exhaustion of reduced enzyme co-factors may be a critical event for the activation of the apoptotic pathway in NQO1-expressing cells following β-Lap exposure. We, therefore, measured intracellular ATP and ADP in log-phase MCF-7 cells after various doses and times of β-Lap (using a luciferase-based bioluminescent assay (42)). Intracellular ATP levels were reduced in MCF-7 cells after treatment with β-Lap in a dose- and time-dependent manner (Fig. 5*A*). At all doses of β-Lap above the LD_{50} of the drug (~2.5 μM) in MCF-7 cells (2), intracellular ATP levels were reduced by >85% at 4 h, the time at which drug was removed (Fig. 5*A*, *left*); the loss of ATP correlated well with β-Lap-induced cell death in MCF-7 cells (Fig. 1*C*). ADP levels remained relatively unchanged after various doses of β-Lap, however, the [ATP]/[ADP][P_i] ratio decreased dramatically. Intracellular ATP levels began to drop to 70% of control levels 2 h after 5 μM β-Lap exposure, the time at which β-Lap began to elicit mitochondrial membrane depolarization (Figs. 5, *A, right,* and 4, *c*). ATP levels continued to drop to 8% of control levels by 4 h after drug exposure (Fig. 5*A*, *right*). In contrast, ADP levels remained relatively unchanged during the course of the experiment, with an increase at 30 min (172% control levels) that returned to control levels by 1 h post-treatment. Cellular ATP levels in β-Lap-treated cells did not appear to recover to normal levels within the 6–24-h interval after drug removal (data not shown).

Loss of ATP following β-Lap was prevented by a 30-min pretreatment with an intracellular Ca^{2+} chelator, but not an extracellular Ca^{2+} chelator (Fig. 5*B*). At 4 h, pretreatment with 10 or 30 μM BAPTA-AM elicited only 58 and 43% ATP loss, respectively, compared with β-Lap alone (92% loss). The extracellular Ca^{2+} chelator, EGTA, did not significantly affect the loss of ATP, nor [ATP]/[ADP][P_i] ratio observed in MCF-7 cells after β-Lap treatment (Fig. 5*B*). Exposure of MCF-7 cells to TG (200 nM) did not elicit decreases in ATP or ADP levels 4 h after drug exposure, compared with untreated control cells.

Ca^{2+} Chelators Prevent β-Lap-induced Proteolysis—We previously showed that apoptosis in various breast cancer cell lines induced by β-Lap was unique, causing a pattern of PARP and p53 intracellular cleavage events distinct from those induced by caspase activating agents (12). After β-Lap treatment, we observed an ~60-kDa PARP cleavage fragment and specific cleavage of p53 in NQO1-expressing breast cancer cells. Furthermore, we showed that this proteolysis in β-Lap-treated cells was the result of activation of a Ca^{2+}-dependent protease with properties similar to μ-calpain (12). PARP and p53 proteolysis in β-Lap-exposed, NQO1-expressing cells was prevented by pretreatment with the extracellular Ca^{2+} chelators, EGTA and EDTA, in a dose-dependent manner (at 8 and 24 h) (Ref. 12, and data not shown). Additionally, PARP, p53, and lamin B proteolysis induced at 24 h in MCF-7 cells following β-Lap treatment were abrogated by pretreatment with 10 or 30 μM BAPTA-AM (Fig. 6). These data strongly suggest that a Ca^{2+}-dependent pathway and potentially a Ca^{2+}-dependent protease are operative in β-Lap-mediated apoptosis.

A simple explanation for the aforementioned results could be that BAPTA blocks bioactivation of β-Lap by NQO1 in a manner similar to that of dicumarol (5). However, BAPTA (free acid) did not affect the enzymatic activities of NQO1 using standard enzymatic assays (data not shown) (5). The free acid (active) form of BAPTA, instead of its −AM ester form, was used in these assays since intracellular accumulation of this Ca^{2+} chelator was not necessary and was physiologically relevant in the *in vitro* enzyme assay. Using β-Lap as a substrate, NQO1 enzymatic activity in the presence of 10 mM BAPTA (a dose of the free acid form of BAPTA that was >1000-fold higher than that used in the experiments of Figs. 1–6) was reduced by <20%. Thus, BAPTA-AM did not affect the activity of NQO1, a two-electron reductase required for β-Lap cytotoxicity (5). We conclude that BAPTA-AM prevents β-Lap-induced apoptosis by blocking Ca^{2+}-mediated signaling events via chelating intracellular Ca^{2+}.

β-Lap Bioactivation by NQO1 Is Critical for Ca^{2+}-mediated Signaling—We previously reported that cells expressing NQO1 are more sensitive to the cytotoxic effects of β-Lap (5).[2] NQO1 is inhibited by dicumarol, which competes with NADH or NADPH for binding to the oxidized form of the enzyme. Dicumarol thereby prevents reduction of quinones (50, 51). We demonstrated that dicumarol attenuates β-Lap-mediated proteolysis of apoptotic substrates (*e.g.* PARP and p53), apoptosis, and survival in NQO1-expressing cells (5).[2] As expected, increases

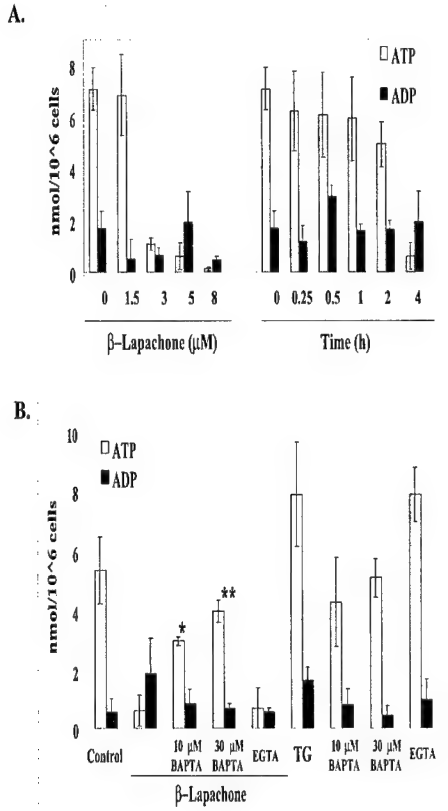

FIG. 5. **ATP depletion after β-Lap treatment is Ca^{2+} dependent.** Intracellular ATP and ADP levels were measured using a luciferase-based bioluminescent assay. *A*, cells were treated with the indicated dose of β-Lap for 4 h or were treated with 5 μM β-Lap for the time indicated, and harvested for ATP analyses. ATP levels were expressed as nanomoles of ATP per 10^6 cells. Purified ATP was used as a standard to determine intracellular ATP concentrations. *B*, cells were either pretreated or untreated with the indicated Ca^{2+} chelators for 30 min prior to drug addition, and β-Lap (5 μM) was then added for 4 h. Cells were harvested for analyses following β-Lap exposure. Results represent the average of at least three independent experiments, ± S.E. Student's *t* test for paired samples, experimental group compared with drug alone are indicated (*, $p < 0.05$; **, $p < 0.01$).

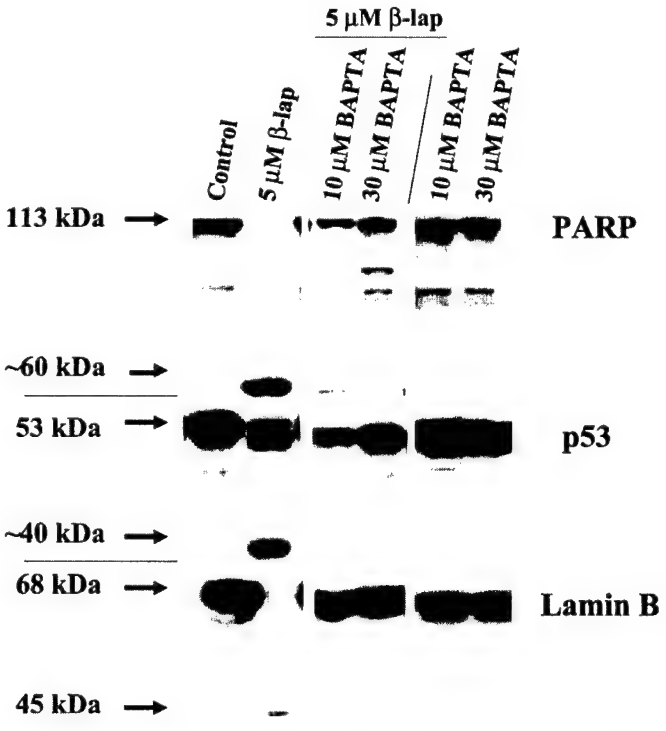

FIG. 6. **Intracellular Ca^{2+} chelators prevent apoptotic proteolysis after β-Lap treatment.** Apoptotic proteolysis was measured in MCF-7 cells exposed to a 4-h pulse of 5 μM β-Lap, with or without a 30-min pretreatment of the indicated dose of BAPTA-AM. Whole cell extracts were prepared 24 h after drug addition, and analyzed using standard Western blotting techniques with antibodies to PARP, p53, and lamin B. Shown is a representative Western blot of whole cell extracts from experiments performed at least three times.

in intracellular Ca^{2+} levels in NQO1-expressing human cancer cells elicited by β-Lap were abrogated by co-treatment with 50 μM dicumarol in 26 of 27 cells (96%) examined (Fig. 7*A*, *lower panel*). The ability of dicumarol to inhibit increases in intracellular Ca^{2+} levels was greater than that observed with BAPTA-AM, where intracellular Ca^{2+} level increases were prevented in only 89% of cells examined (Fig. 3*B*). Thus, NQO1 was critical for the rise in intracellular Ca^{2+} levels observed in MCF-7 cells after β-Lap exposure.

Mitochondrial membrane depolarization induced by β-Lap was also abrogated by pretreatment with dicumarol (Fig. 7*B*). By 4 h, the majority of β-Lap-treated cells exhibited low mitochondrial membrane potential (58%), while very few control cells were depolarized (9%) (Fig. 7*B*). Pretreatment with dicumarol attenuated this response to β-Lap, with only 34% being depolarized. The inability of dicumarol to prevent mitochondrial depolarization in 34% of β-Lap-treated cells was probably due to the high background of control cells (20%) that were depolarized after exposure to dicumarol alone. In comparison with intracellular Ca^{2+} buffering, BAPTA-AM elicited only a minor depolarization of the mitochondria on its own (low, 14%) and thus was able to elicit a greater protective effect (Fig. 4*B*); only 23% of cells exposed to BAPTA-AM and β-Lap exhibited low mitochondrial membrane potential as compared with β-Lap exposed cells in the presence of dicumarol (34%).

The dramatic loss of intracellular ATP in MCF-7 cells following β-Lap exposure was inhibited by a 30-min pretreatment with 50 μM dicumarol (Fig. 7*C*). β-Lap-treated MCF-7 cells pretreated with dicumarol exhibited only 34% loss of intracellular ATP, compared with 92% loss after β-Lap treatment alone (Fig. 7*C*). ADP levels were not altered by any of the treatments used, however, the [ATP]/[ADP][P_i] ratio decreased dramatically in β-Lap-treated cells, and was only partially decreased with dicumarol pretreatment alone, as compared with control untreated cells.

Dicumarol also abrogated DNA fragmentation induced by β-Lap in MCF-7 cells. MCF-7 cells exhibited 94% apoptosis following β-Lap exposure that was prevented by a 30-min pretreatment with 50 μM dicumarol; only 6% of the cells staining positive in a TUNEL assay at 24 h post-treatment (Fig. 7*D*). These data are consistent with prior results (5), and correlate well with the survival protection afforded by dicumarol to β-Lap-treated cells. Dicumarol did not induce DNA fragmentation on its own. These data are consistent with the protection from apoptosis observed with either intra- and extracellular Ca^{2+} chelators. BAPTA-AM or EGTA protected β-Lap exposed MCF-7 cells from apoptosis (Fig. 1, *A* and *B*). Collectively, these data implicate the bioactivation of β-Lap by NQO1 as a critical step in the rise of intracellular Ca^{2+} levels following β-Lap exposure, and thus β-Lap-mediated downstream apoptotic events.

DISCUSSION

When homeostatic mechanisms for regulating cellular Ca^{2+} are compromised, cells may die, either by necrosis or apoptosis (20, 21, 36). We demonstrated that bioactivation of β-Lap by NQO1 induced cell death in a manner that was dependent upon Ca^{2+} signaling (Figs. 1–6). β-Lap can be reduced by NQO1 and

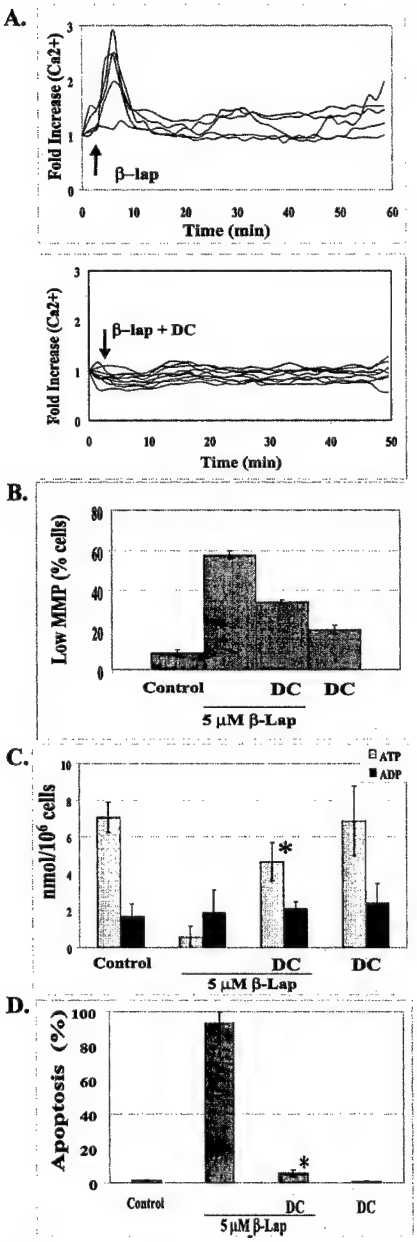

FIG. 7. **NQO1-dependent activation of β-Lap is critical for Ca^{2+} signaling.** *A,* intracellular Ca^{2+} was measured on live cells using the Ca^{2+} indicator dye, fluo-4-AM, and confocal microscopy as described in the legend to Fig. 3. Three basal images were recorded before drug treatments. β-Lap (8 μM) was then added to MCF-7 cells, either alone (*upper panel*) or in combination with 50 μM dicumarol (*lower panel*). Images were collected every 90 s for 50–60 min. Shown are representative graphs displaying changes in fluo-4 fluorescence for the duration of the experiment. Each *line* represents the fold change in fluo-4 fluorescent emission (as compared with basal levels) of an individual cell from one experiment, and the graph is representative of experiments performed at least three times. *B,* mitochondrial membrane potential was measured using the JC-1 dye as described in the legend to Fig. 4. MCF-7 cells were treated with 50 μM dicumarol 30 min prior to β-Lap exposure. Four hours later, cells were harvested for analyses of mitochondrial membrane potential. Shown are mean ± S.E. of the percentage of cells with low mitochondrial membrane potential of at least two independent experiments. *C,* ATP and ADP levels were assayed as described in the legend to Fig. 5. Cells were pretreated with dicumarol for 30 min prior to drug addition, 5 μM β-Lap was added for 4 h, and cells were harvested immediately thereafter for analyses. Results represent the mean of at least three independent experiments ± S.E. Student's *t* test for paired samples, experimental groups compared with drug alone are indicated (* $p < 0.05$). *D,* apoptosis, using the TUNEL assay, was assessed as per Fig. 1. MCF-7 cells were treated with 50 μM dicumarol 30 min prior to a 4-h exposure of 5 μM β-Lap. Cells were then harvested for TUNEL analyses at 24 h post-treatment. Shown are mean ± S.E. of at least three independent experiments. Student's *t* test for paired samples, experimental groups compared with β-Lap exposure alone are indicated (*, $p < 0.005$). *DC,* 50 μM dicumarol.

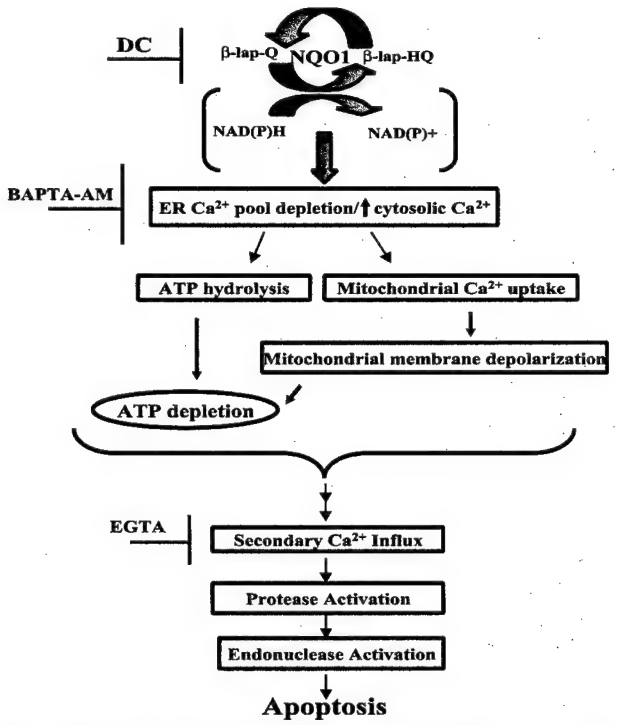

FIG. 8. **Proposed model for β-lapachone-mediated apoptosis in NQO1-expressing cells.** In cells that express NQO1, β-Lap is reduced from the quinone (*β-lap-Q*) to the hydroquinone (*β-lap-HQ*) form in a futile cycle that results in dramatic losses of NAD(P)H (5). During the metabolism of β-Lap by NQO1, Ca^{2+} is subsequently released from the ER causing a rise in cytosolic Ca^{2+} levels by an as yet unknown mechanism. To maintain low cytoplasmic Ca^{2+} levels, we theorize that mitochondria sequester Ca^{2+} and numerous cellular ATPases probably function to pump Ca^{2+} out of the cytosol. This leads to mitochondrial membrane depolarization and ATP hydrolysis, respectively (Figs. 4 and 5). Sustained depolarization of the mitochondrial membrane leads to further loss of ATP and prevents ATP synthesis by inhibiting respiration. The loss of ATP disrupts ionic homeostasis within the cell and thereby allows extracellular Ca^{2+} to enter the cell down its concentration gradient (see "Discussion"). The secondary rise in cytosolic Ca^{2+} levels leads to protease (presumably activation of calpain or a calpain-like protease) and, thus, endonuclease (DFF40) activation, ultimately resulting in apoptosis.

may undergo futile cycling between quinone and hydroquinone forms (β-Lap-Q and β-Lap-HQ, Fig. 8), presumably depleting NADH and/or NADPH in the cell (5). We theorize that depletion of NAD(P)H, along with a rise in intracellular Ca^{2+} levels in response to β-Lap, activate a novel caspase-independent apoptotic pathway, as described in this paper and previously (2, 5, 12). The rise in intracellular Ca^{2+} appears to be dependent upon the bioactivation of β-Lap by NQO1, suggesting a critical and necessary signaling role for Ca^{2+} in the downstream apoptotic pathway induced by this drug. Dicumarol completely abrogated intracellular Ca^{2+} changes (Fig. 7), as well as apoptosis and survival, following β-Lap exposure of NQO1-expressing cells (5).[2] When increases in intracellular Ca^{2+} levels were directly prevented by pretreatment with BAPTA-AM, downstream apoptotic responses, as well as lethality, caused by β-Lap were prevented; when corrected for BAPTA-AM affects alone, β-Lap-induced apoptosis, proteolysis, and lethality were essentially blocked by preventing early Ca^{2+} release from ER stores. Thus, correcting for the BAPTA-AM affects alone, the role of Ca^{2+} in β-Lap-mediated apoptosis may be more significant that that revealed by the data shown. These data strongly

suggest that DNA fragmentation, mitochondrial membrane depolarization, ATP loss, and apoptotic proteolysis were a consequence of the increase in intracellular Ca^{2+} levels (Figs. 1–6 and 8). Interestingly, the cell death pathway induced by β-Lap was quite distinct from that observed after exposure to TG, an agent known to specifically cause release of Ca^{2+} from ER stores and mediate caspase-dependent apoptosis (24, 28, 33, 52). Thus, Ca^{2+} release was necessary for β-Lap-induced cytotoxicity, but apparently not sufficient for the unique apoptotic responses induced by β-Lap.

β-Lap and TG-induced Similar Ca^{2+} Responses, but Different Patterns of Apoptosis—β-Lap elicited an early rise in intracellular Ca^{2+} levels from the same ER store as released by TG, however, subsequent cell death processes were remarkably different between the two compounds. TG is known to cause transient increases in intracellular Ca^{2+} levels, however, these were insufficient to induce apoptosis. Much like β-Lap, Ca^{2+} was needed from the extracellular milieu, along with a sustained increase in intracellular Ca^{2+} levels, for TG-induced apoptosis (23) in MCF-7 cells (27). Depolarization of the mitochondrial membrane potential and loss of intracellular ATP in cells exposed to β-Lap, may have prevented plasma membrane Ca^{2+} pumps and ER Ca^{2+} pumps from functioning and maintaining Ca^{2+} homeostasis. This, in turn, may have facilitated Ca^{2+} leakage down its concentration gradient into the cytosol, providing a secondary and sustained elevation of Ca^{2+} that initiated a protease cascade(s) and ultimately caused apoptosis after exposure to β-Lap. This is consistent with what we observed in NQO1-expressing cells after β-Lap treatment and co-administration of Ca^{2+} chelators. Buffering intracellular Ca^{2+} with BAPTA-AM partially abrogated all of the downstream events induced in MCF-7 cells by β-Lap (and thus prevented secondary Ca^{2+} entry by buffering the initial rise in cytosolic Ca^{2+}). In contrast, extracellular chelation by EGTA only prevented those events initiated by secondary Ca^{2+} entry (*e.g.* protease activation and DNA fragmentation). Thus, a secondary rise in intracellular Ca^{2+} levels after exposure to β-Lap seems probable, and necessary, for protease activation and DNA fragmentation as was observed for TG-induced caspase-mediated apoptosis (23, 27). However, a secondary influx of Ca^{2+} does not appear to be necessary for reduction in mitochondrial membrane potential or loss of intracellular ATP after β-Lap exposure, since EGTA did not prevent these responses.

Although MCF-7 cells treated with β-Lap had similar calcium responses, as do TG-exposed cells, β-Lap-exposed cells exhibited a very different pattern of apoptosis than TG-treated cells. β-Lap-exposed cells exhibit loss of intracellular ATP and a decrease in the [ATP]/[ADP][P_i] ratio. In contrast, TG-exposed cells did not exhibit loss of ATP (Fig. 5, and as reported by Ref. 53). Our data suggest that in contrast to TG where ATP-dependent caspase activation results in cell death (28, 33, 34, 54), an ATP-independent protease is activated after exposure to β-Lap. Ca^{2+} may regulate apoptosis by activating Ca^{2+}-dependent protein kinases and/or phosphatases leading to alterations in gene transcription. However, with the rapid loss of intracellular ATP after exposure to β-Lap (2–4 h, Fig. 5), β-Lap-mediated cell death unlikely involves stimulated kinases or phosphatases or new protein synthesis. Instead, indirect kinase inhibition, due to ATP depletion, along with continued phosphatase activity is likely. Consistent with this notion, we found dramatic de-phosphorylation of pRb in cells exposed to β-Lap at 3 h (2), a time consistent with loss of ATP following exposure to this drug. Furthermore, loss of ATP at 2 h may also be responsible for inhibition of NF-κB activation induced by tumor necrosis factor-α in β-Lap pre-exposed cells (55), since significant loss of ATP would prevent proteosome-mediated IκB degradation. Thus, Ca^{2+}-dependent loss of ATP in NQO1-expressing cells following β-Lap treatment may explain the reported pleiotropic effects of this agent.

β-Lap-exposed cells also exhibited a very different pattern of substrate proteolysis compared with that observed after TG (2, 12, 28). We previously showed that β-Lap elicited a unique cleavage of PARP (~60-kDa fragment), compared with the classical caspase-3-mediated fragmentation of the protein (~89 kDa) observed after TG exposure (data not shown and Ref. 28). In a variety of NQO1-expressing cells exposed to β-Lap, atypical PARP cleavage was inhibited by the global cysteine protease inhibitors, iodoacetamide and *N*-ethylmaleimide, as well as the extracellular Ca^{2+} chelators, EGTA and EDTA (12). In addition, β-Lap-mediated apoptotic responses were insensitive to inhibitors of caspases, granzyme B, cathepsins B and L, trypsin, and chymotrypsin-like proteases (12). In contrast, classic caspase inhibitors blocked TG-induced caspase activation and apoptosis (28). Caspase activation, as measured by procaspase cleavage via Western blot analyses, does not occur following β-Lap exposures.[3] Thus, protease activation after β-Lap treatment appears to be Ca^{2+}-dependent, or alternatively, is activated by another protease or event that is Ca^{2+}-dependent (Figs. 1–6 and Ref. 12).

Loss of Reducing Equivalents Is Also Necessary for β-Lap-mediated Apoptosis, Similar to Menadione-mediated Apoptosis—Menadione is a quinone that can be detoxified by NQO1 two-electron reduction. However, menadione can also be reduced through two, one-electron reductions via other cellular reductases (56), thus eliciting menadione's toxic effects. Menadione toxicity, elicited via two, one-electron reductions, exhibited many similarities to β-Lap-mediated, NQO1-dependent, toxicity (5). These included: (*a*) elevations in cytosolic Ca^{2+} (57, 58); (*b*) NAD(P)H depletion (5, 59, 60); (*c*) ATP depletion (<0.1% control)[3] (61–63); and (*d*) mitochondrial membrane potential depolarization[3] (64). We previously demonstrated that menadione caused similar substrate proteolysis (p53 and atypical PARP cleavage) in NQO1-deficient cells, or at high doses in cells that express NQO1 where detoxification processes were over-ridden (5).[3] The semiquinone form of menadione can undergo spontaneous oxidation to the parent quinone (59, 63, 65, 66); a pattern similar to the futile cycling observed after β-Lap bioactivation by NQO1 (5). Loss of reducing equivalents, such as NADH, due to the futile cycling of menadione may cause inactivation of the electron transport chain with the concomitant loss of mitochondrial membrane potential, and thus, loss of ATP (67, 68). These responses were also observed in MCF-7 cells exposed to β-Lap (Figs. 4 and 5). Extensive mitochondrial Ca^{2+} accumulation can also mediate mitochondrial depolarization (69, 70). Thus, Ca^{2+} sequestration may elicit mitochondrial membrane depolarization and consequent ATP depletion in cells exposed to β−lap. These data further suggest that Ca^{2+} is necessary for β-Lap-mediated cell death, but other factors are apparently needed for the initiation of the novel execution apoptotic pathway observed in cells treated with this compound.

The rise in intracellular Ca^{2+} appears to be dependent on the bioactivation of β-Lap by NQO1, suggesting a critical and necessary signaling role for Ca^{2+} in the downstream apoptotic pathway induced by this drug. These data suggest that DNA fragmentation, mitochondrial membrane depolarization, ATP loss, and apoptotic proteolysis were a consequence of the increase in intracellular Ca^{2+} levels. Work in our laboratory is focused on elucidating the signaling response(s) that elicits ER Ca^{2+} release following β-Lap bioactivation by NQO1. The cell

[3] C. Tagliarino, J. J. Pink, and D. A. Boothman, unpublished results.

death pathway induced by β-Lap is quite distinct from that observed after exposure to TG, and β-Lap-mediated apoptosis exhibited many similarities to menadione-mediated apoptosis. These observations further suggest that early release of Ca^{2+} from ER stores, as well as influx of Ca^{2+} from the extracellular milieu are necessary, but not sufficient for the novel apoptotic execution pathway induced by β-Lap. Thus, changes in Ca^{2+} homeostasis in conjunction with the presumed loss of reducing equivalents are both necessary and sufficient for β-Lap-mediated apoptosis. We propose that development of β-Lap for treatment of human cancers that have elevated NQO1 levels (*e.g.* breast and lung) is warranted (6). Since most clinical agents used to date kill cells by caspase-dependent and p53-dependent pathways, and many cancers evade death by altering these pathways, development of agents that kill by specific targets (NQO1-mediated) and in p53- and caspase-independent manners are needed.

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μ-Calpain Activation in β-Lapachone-Mediated Apoptosis

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Running Title: μ-Calpain Activation in β-Lap-Mediated Apoptosis

Summary

β-Lapachone (β-Lap) triggers apoptosis in a number of human breast and prostate cancer cell lines through a unique apoptotic pathway that is dependent upon NQO1, a two-electron reductase. Recently, our laboratory showed that β-lap-exposed MCF-7 cells exhibited an early increase in intracellular cytosolic Ca^{2+} from endoplasmic reticulum stores, and that BAPTA-AM (an intracellular Ca^{2+} chelator) blocked these early increases and inhibited all aspects of β-lap-induced apoptosis. We now show that exposure of NQO1-expressing breast cancer cells to β-lap stimulates a unique proteolytic apoptotic pathway mediated by μ-calpain activation. Upon activation, μ-calpain translocated to the nucleus concomitant with specific nuclear apoptotic-proteolytic events. The apoptotic responses in NQO1-expressing cells to β-lap were significantly delayed and survival enhanced via exogenous expression of calpastatin, a natural inhibitor of μ- and m- calpains. Furthermore, μ-calpain cleaved PARP to a unique fragment (~60 kDa), not previously reported for calpains. We provide evidence that β-lap-induced, μ-calpain-stimulated apoptosis does not involve any known apoptotic caspases; the activated fragments of caspases were not observed after β-lap exposures, nor were there any changes in the pro-enzyme forms as measured by Western blot analyses. The ability of β-lap to trigger an apparently novel, p53-independent, calpain-mediated apoptotic cell death further support the development of this drug for improved breast cancer therapy.

Keywords: β-Lapachone, apoptosis, Ca^{2+}, calpain, breast cancer

Introduction

β-Lap[1] is a naturally occurring quinone present in the bark of the South American Lapacho tree. The drug has antitumor activity against a variety of human cancers, including colon, prostate, promyelocytic leukemia and breast (1-3). β-Lap was shown to be an effective agent (alone and in combination with taxol) against human ovarian and prostate xenografts in mice, with low level host toxicity (4). We recently demonstrated that β-lap killed human breast and prostate cancer cells by apoptosis, a cytotoxic response significantly enhanced by NAD(P)H:quinone oxidoreductase (NQO1, E.C. 1.6.99.2) enzymatic activity (5,6). β-Lap cytotoxicity was prevented by co-treatment with dicumarol (an NQO1 inhibitor) in NQO1-expressing breast and prostate cancer cells (5,6). NQO1 is a cytosolic enzyme elevated in breast cancers (7) that catalyzes a two-electron reduction of quinones (e.g., β-lap, menadione), utilizing either NADH or NADPH as electron donors. We recently showed that reduction of β-lap by NQO1 presumably leads to a futile cycling of the compound, wherein the quinone and hydroquinone form a redox cycle with a net concomitant loss of reduced NAD(P)H (5). We also demonstrated that increases in intracellular Ca^{2+} levels were critical for the apoptotic pathway induced by β-lap (8). Increased cytosolic Ca^{2+}, due to ER Ca^{2+} pool depletion, led to loss of mitochondrial membrane potential, ATP depletion, specific and unique substrate proteolysis, DNA fragmentation and cell death by apoptosis (8).

Apoptosis is an evolutionarily conserved pathway of biochemical and molecular events that underlie certain cell death processes involving the activation of intracellular zymogens. Once apoptosis is initiated, biochemical and morphological changes occur irreversibly that commit the cell to die. These changes include: DNA fragmentation, chromatin condensation, cytoplasmic membrane blebbing, cleavage of apoptotic substrates (e.g., PARP, lamin B), and

loss of mitochondrial membrane potential with concomitant release of cytochrome c into the cytoplasm (9-11). Apoptosis is a highly regulated, active process that requires the participation of endogenous proteases that systematically dismantle the cell. The most well-characterized proteases in apoptosis are caspases, aspartate-specific cysteine proteases, that work through a cascade initiated by either (a) mitochondrial membrane depolarization leading to the release of cytochrome c and Apaf-1 into the cytoplasm (12), that then activates caspase 9 (13) or (b) activation of receptor-mediated caspase activation complexes (e.g., TRADD/MORT1) that initiate caspase pathways primarily via the activation of caspases 8 and 10 (reviewed in 14). In contrast, non-caspase-mediated pathways are less understood and specific activation pathways have only been superficially described.

Calpains are a family of cysteine proteases existing primarily in two forms designated by the Ca^{2+} concentration needed for activation *in vitro,* μ- calpain (calpain-I) and m-calpain (calpain-II). Each form is a heterodimer consisting of a large catalytic and a small regulatory subunit that is activated by increased Ca^{2+} concentrations. Calpains are predominantly located in the cytoplasm (15,16), but can translocate to cellular membranes where they can become activated (17). Calpains have been implicated in neutrophil apoptosis, glucocorticoid-induced thymocyte apoptosis, and adipocyte differentiation, but their patterns of activation are not well characterized (18-20).

Inhibition of calpain can be separated into endogenous and exogenous classifications, and can be used to prevent downstream calpain proteolytic reactions *in vitro* and *in vivo.* Calpastatin is the specific endogenous inhibitor of m- and μ- calpains and binds the catalytic subunits of calpains when associated with the regulatory subunit and only in the presence of Ca^{2+} (21,22). Calpastatin is a specific-competitive inhibitor of m- and μ- calpain and not the muscle-specific

calpain p94, nor other known proteases (e.g., papains, trypsin) (23-27). Calpastatin inhibits activation and expression of the catalytic activity of m- and μ- calpains in a substrate-competitive manner and is itself a substrate of calpains (26-29). Binding of calpains to calpastatin is reversible and does not result in any lingering loss of calpain activity (30). Calpastatin contains four repetitive and homologous domains (1-4) and one non-homologous sequence on the amino-terminus (L-domain) (31-33). Domains 1-4 each inhibit both m- and μ-calpain; each domain is a functioning unit of calpastatin and can bind one calpain molecule and inhibit its activity (25,34-36). Each domain has three conserved sequences, designated N, M, and C. The middle M domain interacts with the active site of calpain and is essential for inhibiting the proteolytic activity of calpains (27), but does prevent the interaction of calpain with membranes (37). A peptide inhibitor (CN) containing the M sequence is another way to inhibit calpain activity specifically (25,38). Other inhibitors have been used to inhibit calpain activity, however, use of these inhibitors are less useful due to their poor solubilities, intracellular uptake, and non-specific ability to inhibit other proteases and/or the proteosome (e.g., cathepsins B and L, papains, etc.) (39-43).

We previously demonstrated that apoptosis in human cancer cells following β-lap administration was unique, in that an ~60 kDa cleavage fragment of poly (ADP-ribose) polymerase (PARP), as well as a distinct intracellular proteolytic cleavage of p53, were observed in NQO1-expressing breast or prostate cancer cells (5,6,8). These cleavage events were distinct from those observed when caspases were activated by topoisomerase I poisons, staurosporine, or administration of Granzyme B (44,45). Furthermore, β-lap-mediated cleavage events were blocked by dicumarol (an inhibitor of NQO1), global cysteine protease inhibitors, as well as both intra- and extra- cellular Ca^{2+} chelators (8,44). Based on these data, we concluded that β-lap

exposure of NQO1-expressing breast and prostate cancer cells elicited the activation of a Ca^{2+}-dependent protease with properties similar to calpain. In particular, the p53 cleavage pattern of β-lap-exposed cells (5,44) was remarkably similar to the pattern observed after calpain activation (46,47).

In this study, we show that β-lap mediates a unique proteolytic apoptotic pathway in NQO1-expressing cells via μ-calpain activation. Upon activation, μ-calpain translocates to the nucleus where it can proteolytically cleave PARP and p53. We provide evidence that suggests that β-lap-induced, μ-calpain stimulated, apoptosis does not involve any of the known caspases, including caspase 12. Furthermore, the apoptotic responses in NQO1-expressing cells to β-lap can be significantly delayed, and survival enhanced, via the exogenous over-expression of calpastatin, a natural calpain inhibitor.

Experimental Procedures

Reagents- β-Lap (3,4-dihydro-2, 2-dimethyl-2H-naphtho [1,2b] pyran-5, 6-dione) was synthesized by Dr. William G. Bornmann (Memorial Sloan Kettering, NY, NY), dissolved in DMSO at 10 mM, and the concentration verified by spectrophotometric analysis (2,44). Menadione, staurosporine, ionomycin, dicumarol, bovine serum albumin, saponin, and the acetyl-calpastatin peptide inhibitor (CN) were obtained from Sigma Chemical Co. (St. Louis, MO). PARP cDNA was a gracious gift from Dr. Xiaodong Wang (University of Texas Southwestern Medical School, Texas) and was cloned into pcDNA3.1HisB (Invitrogen, Carlsbad, CA). The calpastatin cDNA was cloned into the mammalian expression vector pcDNAI-neo (Invitrogen) and was a gracious gift from Dr. Marc Piechaczyk (Institut de Genetique Moleculaire, Montpellier, France).

Cell Culture- MCF-7:WS8 (MCF-7) human breast cancer cells were obtained from Dr. V. Craig Jordan, (Northwestern University, Chicago, IL). NQO1-deficient MDA-MB-468 (MDA-468) cells were obtained from the American Type Culture Collection and stably transfected with the NQO1 cDNA in the pcDNA3 constitutive expression vector as described (MDA-468-NQ3) (5). Tissue culture components were purchased from Life Technologies, Inc., unless otherwise stated. Cells were grown in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum, in a 37 °C humidified incubator with 5% CO_2 and 95% air atmosphere as previously described (2,44). For all experiments, log-phase MCF-7 breast cancer cells were exposed to 5 μM β-lap for 4 h and MDA-468-NQ3 breast cancer cells were exposed to 8 μM β-lap for 4 h, after which time fresh medium was added and cells were harvested at various times post-treatment.

Stable Transfection- MCF-7 cells were seeded at 2.5 X 10^5 cells/well in a 6-well plate and allowed to attach overnight. The following day, 1.0 μg of plasmid DNA containing the human calpastatin cDNA in the pcDNAI-Neo constitutive expression vector was transfected into each well using Effectene, as per manufacturer's instructions (Qiagen, Valencia, CA). One day later, cells were selected for growth in 250 μg/ml Geneticin ® (G418, Life Technologies, Inc.). A stable, pooled population was established after approximately three weeks, and subsequent clones were isolated by limiting dilution cloning, as described (48).

Western Blot Analyses- Whole cell extracts from DMSO control or β-lap-exposed breast cancer cells were prepared and analyzed by SDS-PAGE-Western blot analyses as previously described (2,44). Loading equivalence and transfer efficiency were monitored by Western blot analyses of proteins known to be unaltered by β-lap exposures (2); Ponceau S staining of the membrane was also used to verify loading. Probed membranes were then exposed to X-ray film

for an appropriate time and developed. Dilutions of 1:7,000 for the C2-10 anti-PARP antibody (Enzyme Systems Products; Livermore, CA), 1:2,000 for anti-p53 DO-1, anti-cyclin D1 HD11, and anti-lamin B (Santa Cruz; Santa Cruz, CA) antibodies, 1:1,500 for anti-calpain small subunit MAB3083, anti-Calpastatin MAB3084, anti-m-calpain AB1625, anti-μ-calpain MAB3104 (Chemicon, Temecula, CA), and 1:2,000 for anti-actin (Amersham Pharmacia Biotech, Piscataway, NJ) were used as described (2,44). The specificity of the calpain antibodies used were evaluated by Western blot analyses; antibodies to the catalytic subunit, anti-m-calpain AB1625, and anti-μ-calpain MAB3104, only recognized one polypeptide band, and the m-calpain antibody did not cross-react with the activated fragment of μ-calpain, nor did it exhibit an activated fragment of its own (data not shown, Figs. 1-2).

Caspase Activation- Whole cell extracts from DMSO control, β-lap-exposed, staurosporine (STS), or topotecan (TPT)-exposed MDA-468-NQ3 cells were prepared and analyzed by SDS-PAGE-Western blot analyses for specific loss of caspase pro-enzyme forms. The formation of the corresponding cleavage fragment, indicative of active caspase enzymes was also examined where applicable. Cells were exposed to a 4 h pulse of 8 μM β-lap or continuous exposure of 1 μM STS or 10 μM TPT for 24 h. Dilutions of 1:1,500 for anti-caspase 3 and anti-caspase 7 B-94-1 (Pharmingen, San Diego, CA), 1:1,500 for anti-caspase 8 1H10E4H10 and anti-caspase 10 25C2F2 (Zymed, San Fransico, CA), 1:1,000 for anti-caspase 6 K-20 (Santa Cruz, Santa Cruz, CA), 2.0 μg/ml for anti-caspase 9 96-2-22 (Upstate Biotechnology, Lake Placid, NY), and 1.0 μg/ml for anti-caspase 12 NT (Exalpha Biologicals, Boston, MA).

TUNEL Assay- MCF-7 and MDA-468-NQ3 cells were seeded at 1 x 10^6 cells per 10 cm^2 petri dish. Log-phase cells were then treated for 4 h with β-lap, as described above. Medium was collected from experimental as well as control conditions at the times indicated, and attached

along with floating cells were monitored for apoptosis using TUNEL 3'-biotinylated DNA end labeling via the APO-DIRECT kit (Pharmingen, San Diego, CA) as described (5). Apoptotic cells were analyzed and quantified using an EPICS XL-MCL flow cytometer that contained an air-cooled argon laser at 488 nm, 15 mW (Beckman Coulter Electronics; Miami, Fl) and XL-MCL acquisition software provided with the instrument.

Calpain Cleavage Assay- PARP protein was translated using an *in vitro* transcription and translation TNT-coupled Reticulocyte Lysate system (Promega, Madison, WI) according to manufacturer's instructions. ^{35}S-Methionine-labeled protein (1-2 µl) was incubated with 100 µM $CaCl_2$, and either 0.05 U of recombinant human erythrocyte µ-calpain (Calbiochem, San Diego, CA), 20 U of recombinant caspase 3 (BioMol, Plymouth, PA), or 30 µg of cell lysate, unless otherwise indicated. Reactants were incubated at 37 °C for 1 h. The reaction mix was then treated 1:1 with 2X SDS-loading buffer, boiled for 5 min at 90 °C, proteins separated on a 9% SDS-PAGE gel. Gels were fixed and proteins were visualized by autoradiography. MCF-7 or MDA-468-NQ3 cells were separately treated for 4 h with β-lap (as indicated above), 25 µM Menadione, or 10 µM ionomycin. Cells were harvested at 8 h or 10 h, respectively, in lysis buffer (10 mM HEPES pH 7.4, 5 mM $MgCl_2$, 42mM KCl and 0.32 M sucrose) unless otherwise indicated, and sonicated. Protein concentrations were determined using Bradford assays (Bio-Rad, Hercules, CA) using BSA as a standard, as described (49).

Colony-forming Assays- Cells were seeded into 60-mm dishes (2000 cells/dish in duplicate) and allowed to attach overnight to initiate log-phase growth. Cells were then exposed to a 4 h pulse of β-lap (1.5 µM or 3 µM) or DMSO for a control. Medium was removed, fresh medium was added, and cells were allowed to grow for 7 days. Plates were then washed and

stained with crystal violet in 50% methanol. Colonies of >50 normal-appearing cells were then counted and data graphed and analyzed as described (5,6).

Confocal Microscopy- Cells were plated on a 6-well microscope slide and allowed to attach and reach log-phase growth. MCF-7 or MDA-468-NQ3 cells were treated for 4 h with 25 μM menadione, 5 μM or 8 μM β–lap, respectively, or continuously with 1 μM STS or 3 μM ionomycin. Where indicated, dicumarol (50 μM) was concomitantly added with β-lap for 4 h. MCF-7 or MDA-468-NQ3 cells were fixed at 8 h or 10 h, respectively, in 3.7% formaldehyde for 5 min. Cells were left overnight at 4 °C in PBS and indirect immunofluorescent staining was performed after cells were permiabilized in 100% methanol for 10 min at –20°C. Cells were incubated in primary antibody for 2 h at 37 °C at dilutions of 1:100 for anti-calpain 9A4H8D3 (Alexis Corp., San Diego, CA), 1:75 for anti-calpastatin MAB3084 (Chemicon, Temecula, CA), and 1:50 anti-NQO1 that was contained in medium from mouse hydridoma clone A180 (50); all antibodies were diluted in 1% BSA and 0.005% saponin in PBS. Cells were then washed twice for 30 min each in 0.005% saponin in PBS and incubated for 1 h at room temp in secondary FITC-anti-mouse (Vector, Burlingame, CA) at a dilution of 1:50 in antibody diluting buffer. Cells were then washed twice for 30 min each in wash buffer and allowed to sit overnight in fresh wash buffer at 4°C. Slides were coated with mounting medium containing propidium iodide (Vector, Burlingame, CA) for 5 min and imaged with a Bio-Rad MRC-600 confocal microscope (Hercules, CA) equipped with a 63X N.A. 1.25 oil immersion plan-neofluorar objective at room temp. Confocal images were collected using dual excitation at 488 nm and 568 nm from a krypton/argon laser. The epitope for the μ-calpain antibody recognizes amino acids 465-520 (domain III of the catalytic subunit) of human μ-calpain; domain III is the least homologous domain between μ- and m-calpain. The μ-calpain antibody did not cross-react with

m-calpain or calpastatin, nor did the m-calpain antibody cross-react with μ-calpain, since there was no change in m-calpain immunoreactivity after drug exposures (see results).

Results

Exposure of MCF-7 Cells to β-Lap Resulted in μ-Calpain Activation in a Temporal Manner Corresponding to Cell Death- Log-phase MCF-7 cells were treated for 4 h with 5 μM β-lap, at which time fresh medium was applied. Cells were harvested at the indicated times and analyzed for substrate proteolysis, calpain activation (via western blot analyses), and apoptosis. Treatment of MCF-7 cells with β-lap resulted in ~25% apoptotic cells, 4-6 h post-treatment, ~60% apoptosis at 8 h, and >90% apoptotic cells by 24 h, as measured by TUNEL analyzes (Fig. 1A). Treatment of MCF-7 cells with β-lap also resulted in PARP and p53 proteolysis 8 h post-treatment, and complete loss of full-length PARP protein by 16 h post-treatment (Fig. 1B). Proteolysis of the 113 kDa full-length PARP protein to an ~60 kDa cleavage fragment was distinct from that observed after caspase activation (e.g., 89 and 24 kDa fragments) and occurred at the same time p53 was cleaved to a characteristic calpain cleavage fragment of ~40 kDa (Fig. 1B) (46,47,51). Calpain activation was assessed using autolysis of the regulatory as well as the catalytic subunit of μ-calpain (17,47). PARP and p53 underwent proteolysis in a temporal manner corresponding to autolysis of the regulatory subunit of calpain and loss of full-length μ-calpain (Fig. 1B). Calpastatin, an endogenous inhibitor of calpains, was lost by 8 h post-treatment, the same time the small subunit of calpain underwent autolysis and full-length μ-calpain was cleaved (Fig 1B), further implicating calpain activation in β-lap-induced apoptosis. Apoptotic and calpain substrate proteolysis was observed at the same time >50% of cells exposed to β-lap exhibited apoptosis (8 h), as determined by DNA fragmentation measured by the TUNEL assay (Fig. 1A). The other ubiquitously expressed form of calpain, m-calpain, was

not lost (80 kDa) nor cleaved to a fragment (76 kDa) indicative of activation at these times, indicating that m-calpain was not activated in NQO1-expressing cells after β-lap exposures (Fig. 1B). Finally, neither calpain activation nor cell death (loss of survival) occurred in NQO1-deficient cells following 8 μM β-lap treatment (data not shown).

μ-Calpain Activation Occurred Independent of Caspases, in NQO1-expressing Breast Cancer Cells After β-Lap Exposure- Apoptotic pathways have been extensively studied and primarily involve the activation of caspases (52-57). Therefore, we assayed β-lap-exposed NQO1-expressing MDA-468-NQ3 breast cancer cells for caspase activation. Caspase activation was assessed, as previously described, using SDS-PAGE western immunoblot analyses via the formation of the active fragment from the full-length pro-enzyme (Table 1) (58). Since MCF-7 cells lack caspase 3, these studies were performed in caspase 3-containing MDA-468 cells that were transfected with NQO1 to sensitize them to β-lap, as described (5,44). A nominal level of apoptosis was observed 6 h after exposure to β-lap (~15%), increasing to ~38% by 8 h and ~65% by 10 h (Fig. 2A). By 24 h, the percentage of apoptotic cells was >80% (Fig. 2A). Thus, MDA-468-NQ3 cells elicit the same apoptotic proteolysis as MCF-7 cells, but at a later time, 10 h vs. 8 h in MCF-7 cells (Fig. 2B and 1B, respectively). A previously observed lamin B cleavage, as well as p53 cleavage, was observed 10 h after β-lap treatment (Fig. 2B). Proteolysis corresponded to autolysis of both μ-calpain and the small subunit of calpain (Fig. 2B), as well as to the level of detected apoptosis (Fig. 2A).

We then examined known apoptotic caspases for activation 8-24 h following a 4 h 8 μM β-lap exposure. The apoptotic-inducing agents, topotecan and staurosporine (STS), were used as positive controls for caspase activation. None of the caspases examined (3, 6, 7, 8, 9, 10, and 12) nor m-calpain appeared to be activated following β-lap treatment (Table 1). In contrast, μ-

calpain was cleaved to its active form (Table 1). Additionally, the proteolytic events (lamin B, PARP and p53 cleavage events) observed after β-lap treatment were not blocked by the pan-caspase peptide inhibitors zVAD, DEVD, or YVAD ((6,44) and data not shown). Thus, μ-calpain, but not caspase, activation was the predominant event in NQO1-expressing breast cancer cells after β-lap treatment.

Purified μ-calpain Elicited the Same PARP Cleavage Fragment as Found in NQO1-expressing Breast Cancer Cells Exposed to β-Lap- It was previously reported that calpain could cleave PARP in vivo in SH-SY5Y human neuroblastoma cells during maitotoxin-induced necrosis (~40 kDa) (52) or when using purified μ-calpain isolated from calf thymus (~42 kDa, ~55 kDa and ~67 kDa) (59). However, the proteolysis of PARP was not reported to be accompanied by p53 cleavage, nor did it appear to be identical to that observed after β-lap exposures in NQO1-expressing cells (~60 kDa) (44). To directly observe activated μ-calpain activity using PARP substrate, ^{35}S-methionine-labelled PARP was generated by coupled *in vitro* transcription and translation of full-length human PARP cDNA. Labeled PARP protein was then incubated with various cell extracts or purified enzymes. Using this *in vitro* cleavage assay, we examined whether purified μ-calpain could cleave *in vitro* translated ^{35}S-met-PARP to polypeptide fragments as observed in NQO1-expressing breast cancer cells exposed to β-lap. Log-phase MCF-7 or MDA-468-NQ3 cells were treated with β-lap for 4 h and harvested at 8 or 10 h, respectively (corresponding to Western blot analyses of calpain activation and proteolysis after exposure to β-lap, Fig. 1B and 2B), to assess enzymatic activity using ^{35}S-met-PARP. Cell extracts or purified enzyme (μ-calpain or caspase 3, used as a positive control) were incubated with ^{35}S-met-PARP for 1 h, 37°C. Caspase 3 cleaved PARP to the expected 89 and 24 kDa fragments (Figs. 3A-B, lane 2), which were not observed using purified μ-calpain (Figs. 3A-B,

lane 3). In contrast, β-lap-treated cell extracts elicited the same PARP cleavage fragment in a protein concentration- and time- dependent manner, as observed in Western blot analyses (Figs. 3A-B, lanes 4-8 and Figs. 1B and data not shown). The PARP cleavage fragment observed after cell extracts were incubated with ^{35}S-met-PARP was identical to that elicited by recombinant μ-calpain (Figs. 3A, lanes 3 and 4-12 and 3B, lanes 3 and 4-11). Proteolytic activity in cell extracts (control or β-lap-treated conditions) could not be stimulated by addition of Ca^{2+} alone to cell extracts suggesting enzymatic activity was inherent to the β-lap–treated extract upon harvest (data not shown). Menadione-treated cells, the only other known agent to stimulate a similar cell death pathway as β-lap (5,8), were also examined. In contrast to extracts from untreated cells, extracts from menadione-exposed cells caused the cleavage of ^{35}S-met-PARP resulting in fragments similar to those generated by purified μ-calpain or β-lap-treated cell extracts (Figs. 3A, lanes 3, 4-12, and 13 and Fig. 3B, lanes 3, 4-11, and 12). Ionomycin is a calcium ionophore reported to activate calpain (18,60,61). Ionomycin treated MDA-468-NQ3 cell extracts also elicited the same PARP cleavage pattern (Fig. 3B, lanes 13-14), as did μ-calpain, menadione and β-lap-exposed cell extracts (Fig, 3B, lanes 3, 12, and 4-11). Interestingly, ionomycin did not elicit PARP cleavage in MCF-7 cells (Fig. 3A, lane 14).

Calpastatin, A Modulator of β-Lap-Mediated Apoptosis Involving Calpain Activation- We observed that MDA-468-NQ3 cells expressed higher levels of endogenous calpastatin than MCF-7 cells (Fig. 4B), and we hypothesized that the levels of this endogenous calpain inhibitor may explain the slower responses of MDA-468-NQ3 cells (in terms of substrate proteolysis and apoptosis) compared to MCF-7 cells that contain far less calpastatin. In addition, the aforementioned data suggested a correlation between PARP and p53 cleavage with μ-calpain activation. To further investigate whether calpain was indeed involved in apoptotic substrate

proteolysis after β-lap-exposure, we examined the effects of a calpastatin peptide containing the inhibitory sequence of calpastatin, as well as stable over-expression of full-length calpastatin in MCF-7 cells, on substrate proteolysis. Calpastatin is a specific endogenous inhibitor of calpains, and has not been demonstrated to inhibit other proteases (e.g., cathepsins, caspases) (62,63). In the *in vitro* cleavage assay, the calpastatin peptide inhibitor blocked purified μ-calpain-mediated ^{35}S-met-PARP cleavage, but not caspase 3-mediated ^{35}S-met-PARP cleavage (Figure 4A, lanes 2-5). ^{35}S-met-PARP cleavage, mediated by β-lap treated cell extracts, was inhibited in a concentration dependent manner by the calpastatin inhibitory peptide (Fig. 4A, lanes 7-10). In contrast, the calpastatin inhibitory peptide did not prevent STS-mediated ^{35}S-met-PARP cleavage (Fig. 4A, lanes 12-13). In Western blot analyses, 8 h after β-lap exposure, the calpastatin peptide inhibitor slightly diminished PARP cleavage and loss of the small subunit of calpain in a dose-dependent manner. Interestingly, the peptide had a greater effect on the enzymatic activity in menadione-treated cells (data not shown).

MCF-7 cells were transfected with full-length calpastatin and clones selected by limiting dilution cloning (Fig. 4). Two clones with varying levels of calpastatin, MCF-7 CN1 (CN1) and MCF-7 CN2 (CN2), were selected for subsequent analyses. The two MCF-7 calpastatin clones selected expressed intermediate levels of calpastatin, when compared to MCF-7 or MDA-468-NQ3 cells. CN1 cells expressed higher levels of calpastatin than MCF-7 or CN2 cells, but less than MDA-468-NQ3 cells. CN2 cells expressed a much lower level of calpastatin than CN1, but a higher level than MCF-7 cells (Fig. 4B). We are uncertain as to why there is a molecular weight difference between endogenous and exogenous calpastatin in the MCF-7 clones compared to MCF-7 parental cells, however, no endogenous calpastatin was detected at the higher molecular weight in the clones (Fig. 4B). We suspect that the size difference is due to

variations either in transcriptional or posttranslational modifications of exogenously expressed calpastatin, as has been previously reported (63).

Log-phase MCF-7, MDA-468-NQ3, CN1 and CN2 cells were treated for 4 h with 1.5 μM or 3 μM β-lap, at which time fresh medium was added. Clonogenic survival was determined 7 days later. At 1.5 μM β-lap, increasing survival after β-lap exposure correlated with the level of calpastatin expressed in each cell line; MCF-7 cells (low calpastatin) exhibited ~30% survival, CN2 cells (intermediate calpastatin) exhibited ~60% survival, and CN1 cells (high calpastatin) exhibited ~100% survival (Fig. 4C). At 3 μM β-lap, no difference in survival was observed indicating that the inhibitory affects of calpastatin could be overcome with higher drug concentrations (Fig. 4C).

To examine proteolysis during β-lap-mediated cell death in CN1 and CN2 cell clones, cells were harvested at the indicated times for Western blot and apoptosis analyses. MCF-7 cells expressed low levels of calpastatin and exhibited ~50% apoptosis at 8 h, increasing to >90% cells staining TUNEL positive at 24 h. In contrast, β-lap-treated CN1 and CN2 cells that possessed higher levels of calpastatin showed less apoptosis than MCF-7 cells at 8, 10 and 12 h after 5 μM β-lap exposure (Fig. 4D). At 24 h, no difference was observed between the cell lines, indicating that the inhibitory affects of calpastatin could be overcome with time following a 5 μM β-lap exposure (Fig. 4D). As expected, STS did not elicit apoptotic DNA fragmentation in any of the caspase-3 deficient MCF-7 cell clones examined (Fig. 4D). Specific proteolytic events in cells with varied calpastatin levels were then examined following β-lap exposure. β-Lap-exposed MCF-7 cells expressed low levels of calpastatin and exhibited loss of full-length PARP protein at 10 h, and complete loss by 16 h. In contrast, β-lap-treated MDA-468-NQ3 cells that possessed higher levels of calpastatin, showed loss of full-length PARP at 14 h and complete

loss of the protein by 24 h (Fig 4E, top and Fig 1A). Loss of PARP protein also corresponded with loss of the full-length, small subunit of calpains (Fig. 4E, top), indicative of calpain activation (17,47). A non-specific ~90 kDa band was apparent after probing with the PARP antibody that did not change in size or amount during the time-course of treatment of MCF-7 and MDA-468-NQ3 cells with β-lap; similar results were previously reported (44). The small subunit of calpain was lost by 10 h in β-lap-exposed MCF-7 cells, and by 24 h in β-lap-exposed MDA-468-NQ3 cells (Fig. 4E, top). In MCF-7 clones stably expressing calpastatin, β-lap-induced apoptosis and calpastatin levels were inversely correlated (Fig. 4E, bottom) as shown in Fig. 4E, top. CN2 cells expressed low levels of calpastatin and exhibited a similar time-course of PARP and small subunit of calpain proteolysis by 10 h, with complete loss by 12 h as that demonstrated in MCF-7 cells. In contrast, CN1 cells that expressed high levels of calpastatin showed a similar more delayed time-course of proteolysis following β-lap exposure to that of MDA-468-NQ3 cells; loss of full-length PARP protein or the small subunit of calpains was observed in 12 h, with complete loss by 24 h (Fig. 4E, bottom). Furthermore, the small subunit of calpain appeared to be lost much earlier in CN1 cells than in MDA-468-NQ3 cells, but still later than MCF-7 or CN2 cells. In contrast to β-lap exposures, the small subunit of calpain was not lost after STS treatment, nor was STS activity affected by calpastatin over-expression. These data strongly suggest that calpastatin levels can influence the responses of NQO1-expressing cells to β-lap-induced, μ-calpain-mediated apoptosis and survival.

μ-Calpain Translocated to the Nucleus in NQO1-expressing Breast Cancer Cells After β-Lap Exposure- PARP and p53 are nuclear proteins and μ-calpain is reported to primarily reside in the cytoplasm (16,64). Confocal microscopic analyses were performed to examine calpain localization after β-lap treatment. Log-phase MCF-7 or MDA-468-NQ3 cells were treated for 4

h with β-lap (5 μM or 8 μM, respectively) and fixed for indirect immunofluorescent staining at 8 h or 10 h, unless otherwise indicated. Cells were stained with a primary antibody specific for μ-calpain and a FITC-conjugated secondary antibody (green). Slides were then mounted in medium containing propidium iodide for DNA/nuclear staining (red). Cells exhibited μ-calpain translocation to the nucleus (Fig. 5A; μ-calpain staining is green, DNA is red, and nuclear translocation of μ-calpain is indicated by yellow fluorescence) at a time concomitant with μ-calpain activation after β-lap treatment (Figs. 1B and 2B). In addition, translocation of μ-calpain to the nucleus was concomitant with the overall loss of calpastatin immunofluorescence after exposure to β-lap (Fig. 5B, f and h). Translocation of μ-calpain was NQO1-dependent, since it did not occur in cells that lacked NQO1 enzymatic activity (data not shown), and was inhibited by co-administered dicumarol, an inhibitor of NQO1 (Fig 5A).

Translocation of μ-calpain to the nucleus in NQO1-expressing, β-lap-treated cells, was a specific event that did not occur due to nuclear membrane breakdown. Proteins residing in the cytoplasm (NQO1) did not translocate to the nucleus (Fig. 5B, j and l), and proteins that reside in the nucleus (Ku70/Ku80) did not diffuse out into the cytoplasm at the times μ-calpain was observed to translocate to the nucleus (data not shown); Ku70/Ku80 is a heterodimeric protein required for nonhomologous DNA double strand break repair (65). Also, consistent with Western blot analyses in Fig. 1, m-calpain immunoreactivity did not change after exposure of NQO1-expressing breast cancer cells to β-lap (data not shown), further implicating μ-calpain activation in β-lap-induced apoptosis.

Other agents (e.g., menadione) that induce the same cell death pathway as β-lap, also exhibited μ-calpain translocation to the nucleus, while agents that elicit caspase-mediated cell death (e.g., staurosporine) showed no change in μ-calpain localization (Fig. 5A). Ionomycin, a

Ca^{2+} ionophore, activated calpain in some cells (18,60,61). Consistent with these data, μ-calpain translocated to nuclei after treatment with ionomycin in MCF-7 and MDA-468-NQ3 cells (Fig. 5A). Translocation of μ-calpain after ionomycin was more prominent in MDA-468-NQ3 cells than in MCF-7 cells, corresponding to the ability of each cell line to elicit PARP cleavage (Fig. 3A-B, lane 14). Menadione elicited a similar cell death pathway as did β-lap. In a similar manner, exposure of cells to menadione caused translocation of μ-calpain to the nucleus at times concomitant with PARP and p53 substrate proteolysis (Fig. 3 and (5)). In contrast to β-lap exposures, there was no observed change in μ-calpain localization after exposure of MCF-7 or MDA-468-NQ3 cells to staurosporine (STS) (Fig. 5A); STS is a protein kinase inhibitor (66) that initiates apoptosis via a caspase-mediated pathway (67). Collectively, these data implicate μ-calpain activation in β-lap-mediated apoptosis and μ-calpain translocation to the nucleus upon its activation. These data are consistent with the essential role of Ca^{2+} release in β-lap-induced apoptosis as previously demonstrated (8,44).

Discussion

Calpains have been implicated in neutrophil apoptosis, glucocorticoid-induced thymocyte apoptosis, and adipocyte differentiation, but their patterns of activation are not well characterized (18-20). Thus, elucidation of calpain activation after exposure of NQO1-expressing breast cancer cells to β-lap, and the potential role of caspases in regulating β-lap-mediated apoptosis was critical to evaluate. μ-Calpain was activated in a temporal manner corresponding to proteolytic cleavage events, apoptosis, and its apparent translocation to the nucleus from the cytoplasm (Figs. 1-5). Known apoptotic caspases (3, 6, 7, 8, 9, 10 and 12) were not activated after treatment of NQO1-expressing cells with β-lap, suggesting a role for μ-calpain in apoptosis independent of caspase activation (Table 1). Other caspases (1, 4, 5, 11 and 13) were not

assayed for activation after β-lap exposures because they are not associated with most apoptotic pathways, but rather, are implicated in inflammation processes (68,69). Killer/DR5, a death-domain containing pro-apoptotic receptor, mRNA was reported to be upregulated after β-lap exposures in colon cancer cell lines, however, neither receptor activation downstream nor caspase activation were assayed in these cells (70). Since caspases 8 and 10 are important for receptor-mediated apoptosis, through heir association with death domains (e.g., FADD, 71), and neither caspase was activated after β-lap exposures in MDA-468-NQ3 cells (Table 1), our data would strongly suggest that receptor-mediated pathways are not involved in β-lap-induced apoptosis. Furthermore, pan-caspase inhibitors did not prevent apoptotic proteolytic substrate cleavages nor cell death induced by β-lap (6,44). In contrast, Ca^{2+} chelators and the endogenous inhibitor of calpains, calpastatin, did prevent and/or delay proteolytic cleavage events, as well as apoptosis induced by β-lap (Ref. 8,44 and Fig. 4), further implicating a role for calpains in apoptosis independent of caspase activation. Together with the rather dramatic loss of ATP in β-lap-exposed cells (8), these data strongly suggest that caspases are not involved in cell death induced by this cytotoxic agent.

We recently demonstrated that in NQO1-expressing breast cancer cells, shortly after β-lap exposure, increases in intracellular Ca^{2+} levels were critical for apoptosis and cell death induced by β-lap (8). Calpain is a Ca^{2+}-activated protease implicated in a number of apoptotic pathways (18,19,43,55,72). Our results indicate μ-calpain activation after β-lap-induced apoptosis by cleavage of the catalytic subunit, as well as the regulatory subunit of calpains, to fragment sizes indicative of calpain activation (Fig. 1-2). Purified μ-calpain was also able to cleave *in vitro* transcribed and translated PARP in an *in vitro* assay to the same fragment size as

cell extracts with activated calpain (Fig. 3). Finally, cleavage of p53 in β-lap-exposed cells further suggested μ-calpain activation as previously described (46,47).

Since many calpain inhibitors used in earlier work have been shown to also inhibit the proteosome, cathepsins, other cysteine proteases, or inhibit entirely different enzymes, for example, a protein tyrosine phosphatase (42,73,74), we used the specific endogenous inhibitor of calpains, calpastatin, to assay calpain's role in β-lap-mediated apoptosis. A peptide comprised of the inhibitory domain of calpastatin was able to block calpain-mediated cleavage of PARP both *in vitro* and *in vivo* (Fig. 4 and data not shown). Stable over-expression of the calpastatin protein in MCF-7 cells, which possess low levels of calpastatin, delayed apoptotic events and protected against cell death at 1.5 μM β-lap in clonogenic assays (Fig. 4). Calpain has two distinct sites for association with calpastatin, one at the active site and the other at the EF-hand domain (17); it is believed that calpain interacts with substrates through these same two sites. Calpastatin inhibits calpain in a substrate-competitive manner (26,27). Binding of calpains to calpastatin is reversible and does not result in any lingering loss of calpain activity (30). Therefore, the ability of calpain activation to overcome the inhibitory effects of calpastatin was not surprising. The delay of calpain activation observed in cells over-expressing calpastatin after β-lap exposure could be due to the dissociation of calpastatin, or the eventual degradation of calpastatin by initially minimal calpain activation, thus explaining the rather moderate survival and apoptotic inhibition caused by this endogenous inhibitor. It is also possible that calpastatin has a lower affinity for calpain than for other calpain substrates. These data suggest a role for calpain in the effector phase of the apoptotic pathway induced by β-lap.

To unambiguously prove the essential role of calpains in β-lap-induced apoptosis, NQO1-expressing cells deficient in calpain enzymatic activity compared with calpain containing

cells after exposure to β-lap would be paramount. Two groups have made calpain knockout mice; Arthur *et al.* disrupted murine *Capn4* (the gene encoding the regulatory subunit of calpains) which eliminated both m and μ-calpain activities (75) and Azam *et al.* deleted *Capn1* (μ-calpain) in mice (76). However, preliminary data with mouse embryonic fibroblasts from the *Capn4* -/- and +/+ mice suggest that these cells lack NQO1 enzymatic activity and further work with these cells needs to be done to assay for β-lap- induced apoptosis in these cells.

Calpain-Mediated Apoptosis after β-Lap-Exposure of NQO1-Expressing Breast Cancer Cells- A role for calpains in apoptosis has been proposed by many groups, however, calpain activation is usually linked upstream or downstream of caspase activation, or in a parallel pathway alongside caspase activation (54,56,77,78). Caspases have been reported to be upstream of calpain activation by cleaving the endogenous inhibitor of calpain, calpastatin, thus allowing for increased calpain activation after apoptotic induction (54,57). Others have reported calpain activation upstream of caspase activation, demonstrating that calpains specifically triggered activation and processing of caspase 7 and 12 in various model systems (53,77). In addition, calpain may be responsible for cleaving the loop region in Bcl-xL and, therefore, turning the anti-apoptotic Bcl-xL molecule into a pro-apoptotic molecule that further promotes caspase activation (53). Calpains can also inactivate caspases by cleavage of the pro-caspase form to proteolytically inactive fragments (52,79). In addition, calpain activation in apoptosis can occur concurrently with, or in the absence of, caspase activation (80-86). In studies described above, we demonstrated that calpains were activated in human breast cancer cells during β-lap-mediated apoptosis, while caspase activation was not apparent (Fig. 1-4, Table 1). In addition, caspase inhibitors did not have any affect on the apoptotic morphology or survival of

NQO1-expressing cells exposed to β-lap (6,44). This may suggest a redundancy of apoptotic proteases, as well as a novel proteolytic pathway for calpains.

Endonuclease activation is a critical step in the apoptotic pathway culminating in DNA fragmentation. Many DNases are known to exist, some of which are involved in apoptosis, consisting of Ca^{2+}/Mg^{2+}-dependent endonucleases (e.g., DNase I, DNase gamma), Mg^{2+}-dependent endonucleases (CAD/DFF40), and the cation-independent endonucleases (DNase II) (87). Caspase 3 usually mediates cleavage of DFF45/ICAD (a DNase inhibitor) allowing for translocation and activation of DFF40/CAD (an endonuclease) that mediates DNA fragmentation (88). Since caspases were not activated in β-lap-mediated apoptosis and DNA fragmentation was observed via the TUNEL assay (Fig. 1-4 and Table 1), three potential pathways for endonuclease activation in β-lap-mediated apoptosis are possible: (1) Calpains directly activate an endonuclease, (2) Calpains activate another protease that then activates an endonuclease, or (3) Calpains are not involved in endonuclease activation, and Ca^{2+} alone may activate an endonuclease. In some apoptotic pathways, calpain inhibitors blocked all aspects of apoptosis, including DNA fragmentation (18,72,89). These data imply that calpain activation led to endonuclease activation either directly, or indirectly, and it is unclear whether this is through activation of a caspase, another protease, or directly by calpains. Our data suggest that either calpain directly activates an endonuclease or calpain activates another protease (independent of caspases) that then activates an endonuclease, since calpastatin over-expression can delay the onset of DNA fragmentation and calpastatin protein levels were inversely proportional to the time and amount of DNA fragmentation observed after β-lap exposure (Fig. 4). Calpastatin should not affect the levels of Ca^{2+} in the cell and thus should not affect endonuclease activation by Ca^{2+} alone. However, calpastatin may inhibit another protease that is involved in

endonuclease activation. We have preliminary evidence that DFF45 was cleaved concomitantly with PARP and p53 cleavages, as well as calpain activation *in vivo* in MDA-468-NQ3 cells exposed to 8 μM β-lap and by purified μ-calpain *in vitro* (Tagliarino *et al.,* unpublished results). These data would suggest that calpain might activate an endonuclease, DFF40/CAD, directly. Others have demonstrated that calpain inhibitors, but not caspase inhibitors, block DNA fragmentation, further implicating a potential role for calpain in endonuclease activation (18,90).

Calpains are not only suggested to be involved in DNA fragmentation (via endonuclease activation), but are also effector proteases that cleave cellular proteins involved in DNA repair (e.g., PARP), membrane associated proteins (e.g., α-spectrin and actin) and other homeostatic regulatory proteins (e.g., cyclin D1, c-FOS, C-JUN, p53) (17,18,46,52,80,81,91-93). Calpain substrate cleavage does not involve a specific primary cleavage site, but rather, is dependent upon the secondary structure of the substrate, making calpains a class of proteases different from the aspartate-specific caspases (81,94-96). We demonstrated that β-lap-induced activation of μ-calpain in human breast cancer cells mediated cleavage of p53 in a manner similar to that previously reported (46,47). Loss of p53 may further promote apoptosis by preventing anti-apoptotic pathways or cell cycle arrest to allow for cell repair. PARP is a caspase 3 substrate, and a widely used indicator of apoptosis when cleaved to a characteristic 89 kDa fragment from its 113 kDa full-length protein. PARP was previously reported to be cleaved by calpains to a 40 kDa fragment during maitotoxin-induced necrosis (52). PARP was also cleaved by calpains purified from calf thymus to ~42 kDa, ~55 kDa (doublet) and ~67 kDa (triplet) fragments (59). Here, we show a novel cleavage of PARP to an ~60 kDa polypeptide fragment, mediated by μ-calpain in β-lap-treated, NQO1-expressing breast cancer cells during apoptosis (Fig. 1-4).

Calpain Translocated to the Nucleus Upon Activation in β-Lap-Induced Apoptosis- Both m- and μ- calpains are predominantly cytoplasmic (64,97-99). However, some immunoreactivity towards calpains has been localized at the cell membrane (100), at cell-substrate attachment plaques in cultured cells (101), at the I-band in muscle cells (102), or around and within the nucleus (47,51,64,103,104). Calpains exhibit diffuse cytoplasmic staining with no change after activation (16). Conversely, calpains have also been shown to undergo redistribution from the cytosol to the plasma membrane upon activation (105). This suggests a complex activation process for calpains that is agent- and cell-type-specific. While calpains have been reported to cleave a number of nuclear proteins (e.g., p53, PARP) there is negligible data that calpains may be localized to the nucleus, and translocation to the nucleus upon activation is only suggested (47,64). Interestingly, one group showed that μ-calpain immunoreactivity transiently accumulated in cell nuclei concomitantly with proteolysis of p53 in late G1 phase (51). Mellgren *et al.* (104) also demonstrated nuclear transport of purified calpains in permeabilized cells; fluorescein-tagged, μ-calpain was transported into nuclei in an ATP-dependent fashion and calpastatin did not block μ-calpain translocation. Furthermore, upon ischemic damage of cultured neurons, calpain was activated and localized to the nucleus, suggesting a nuclear translocation of μ-calpain upon activation (106). Here, we demonstrate the ability of μ-calpain to translocate to the nucleus in NQO1-expressing breast cancer cells after exposure to β-lap (Fig. 5). μ-Calpain translocated to the nucleus concomitantly with its own activation, apoptotic proteolytic cleavage events and DNA fragmentation (Figs. 1-2 and 5). This translocation would allow for the ability of calpain to proteolytically cleave the nuclear substrates PARP and p53, as well as potentially activate an endonuclease.

We demonstrated that μ-calpain was activated in NQO1-expressing breast cancer cells exposed to β-lap. Its activation involved an apoptotic signal transduction pathway leading to cell death independent of caspase activation and sensitive to calpastatin expression (Figs. 1-4 and Table 1). We also demonstrated a novel translocation of μ-calpain to the nucleus upon its activation in β-lap- and menadione- mediated apoptosis (Fig. 5).

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[1]The abbreviations used are: β-Lap, β-Lapachone; MCF-7, MCF-7:WS8; MDA-MD-468, MDA-468; NQO1, NAD(P)H:Quinone Oxidoreductase, DT-diaphorase (E.C. 1.6.99.2); PARP, Poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; ER, endoplasmic reticulum; STS, staurosporine; TPT, topotecan; LD_{50}, 50 % lethal dose; IC_{50}, 50% inhibitory concentration; NF-κB, nuclear transcription factor-kappa B; pRb, retinoblastoma protein.

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Figure Legends

Figure 1. μ-Calpain activation in β-Lap-mediated apoptosis. (A) TUNEL assays were performed to monitor DNA fragmentation at times indicated in MCF-7 cells after a 4 h pulse of 5 μM β-lap. Results are graphically summarized as the average of at three independent experiments, mean +/- S.E. (B) Apoptotic proteolysis was measured in MCF-7 cells exposed to a 4 h pulse of 5 μM β-lap, under identical conditions described in 1A. Whole cell extracts were prepared at the indicated times and analyzed using standard Western blotting techniques with antibodies to PARP, p53, the small subunit of calpains, m-calpain, μ-calpain, calpastatin, and cyclin D1. Shown is a representative Western blot of whole cell extracts from experiments performed at least three times.

Figure 2. μ-Calpain activation, but not caspase activation, during β-Lap-mediated apoptosis in NQO1-expressing MDA-468-NQ3 cells. (A) TUNEL assays were performed to monitor DNA fragmentation in MDA-468-NQ3 cells at the times indicated after a 4 h pulse of 8 μM β-lap. Results are graphically summarized as the average of at three independent experiments, mean +/- S.E. (B) Apoptotic proteolysis was measured in MDA-468-NQ3 cells exposed to a 4 h pulse of 8 μM β-lap. Whole cell extracts were prepared at the indicated times and analyzed using standard Western blotting techniques using antibodies to lamin B, p53, the small subunit of calpains, μ-calpain and actin. Shown is a representative Western blot of whole cell extracts from experiments performed at least three times.

Figure 3. Purified μ-calpain cleaved PARP to the same fragment size as did NQO1-expressing breast cancer cells exposed to β-lap. PARP protein was translated using an *in vitro*

transcription and translation TNT-coupled Reticulocyte Lysate system. ^{35}S-methionine-labeled protein was incubated with 100 μM $CaCl_2$, and either 0.05 U of recombinant human erythrocyte μ-calpain, 20 U of recombinant caspase 3, or 30 μg of cell lysate, unless otherwise indicated. The reaction mix was incubated at 37 °C for 1 h. (A) MCF-7 cells were treated for 4 h with 5 μM β-lap, 25 μM menadione, or 10 μM ionomycin, and harvested at 8 h, unless otherwise indicated. (B) MDA-468-NQ3 cells were treated for 4 h with 8 μM β-lap, 25 μM menadione, or 10 μM ionomycin, and harvested at 10 h post-treatment, unless otherwise indicated. Sample proteins were separated on a 9% SDS-PAGE gel and ^{35}S-met-PARP was visualized via autoradiography.

Figure 4. Calpastatin inhibits or delays substrate proteolysis, apoptosis and survival after exposure to β-lap. (A) ^{35}S-methionine-labeled protein was incubated with 0.05 U recombinant μ-calpain, 20 U recombinant caspase 3, or 30 μg cell lysate. MCF-7 cells were treated for 4 h with 5 μM β-lap or continuously with 1 μM STS and where indicated, with a calpastatin peptide inhibitor, and harvested at 8 h. Samples were run on a 9% SDS-PAGE gel and ^{35}S-met-PARP was visualized via autoradiography. Shown is a representative experiment from experiments performed at least twice. (B) Whole cell extracts were collected and Western blot analyses performed using a calpastatin primary antibody to determine relative calpastatin levels in MCF-7, MDA-468-NQ3, and MCF-7 cells stably-expressing full-length calpastatin. (C) Cells were seeded into 60-mm dishes (2000 cells/dish in duplicate) and allowed to attach overnight. Cells were then exposed to a 4 h pulse of β-lap. Medium was removed, fresh medium was added, and cells were allowed to grow for 7 days. Plates were then washed and stained with crystal violet in 50% methanol. Colonies of >50 normal-appearing cells were then

counted. Results were graphically summarized as the average of at two independent experiments performed in duplicate, mean +/- S.E. Student's *t* test for paired samples, experimental group compared to MCF-7 cells treated with β-lap were indicated (*, $p<0.01$). (D) At times indicated, TUNEL assays were performed to monitor apoptotic DNA fragmentation in cells after a 4 h pulse of 5 μM β-lap, or 24 h after 1 μM STS continuous treatment. Results were graphically summarized as the average of at two independent experiments, mean +/- S.E. Student's *t* test for paired samples, experimental group compared to MCF-7 cells treated with β-lap were indicated (*, $p<0.1$ and **, $p<0.05$). (E) Apoptotic proteolysis was measured in the various cell lines exposed to a 4 h pulse of 5 μM β-lap, or continuous exposure of 1 μM STS (harvested at 24 h). Whole cell extracts were prepared at the indicated times and analyzed using standard Western blotting techniques with antibodies to PARP and the small subunit of calpains. Shown is a representative Western blot of whole cell extracts from experiments performed at least three times with Ponseau S staining of the membrane for protein loading. NS= nonspecific protein banding.

Figure 5. μ-Calpain translocation to the nucleus after exposure to β-lap. (A) MCF-7 or MDA-468-NQ3 cells were treated for 4 h with 25 μM menadione, 5 μM or 8 μM βlap, respectively, or continuously with 1 μM STS or 3 μM ionomycin. Dicumarol (50 μM) was added concomitantly with β-lap for 4 h. MCF-7 or MDA-468-NQ3 cells were fixed at 8 h or 10 h, respectively, for analyses. Indirect immunofluorescent staining of fixed cells was performed using primary antibodies for anti-μ-calpain, anti-calpastatin and anti-NQO1 with secondary FITC-anti-mouse antibody (green). Slides were coated with mounting medium containing propidium iodide for DNA/nuclear staining (red) and analyzed. Confocal images were collected

using dual excitation at 488 nm and 568 nm from a krypton/argon laser. Nuclear translocation was indicted by yellow fluorescence from the merging of red DNA/nuclear staining and green protein staining.

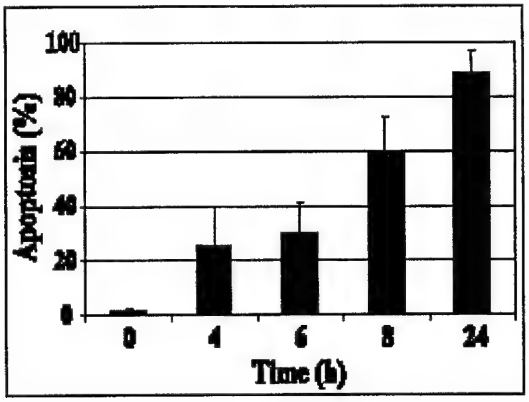
Table 1

Protease	STS	TPT	β-Lap
μ-Calpain	-	-	+
m-Calpain	-	-	-
Caspase 3	+	+	-
Caspase 6	-	+	-
Caspase 7	+	+	-
Caspase 8	+	+	-
Caspase 9	+	+	-
Caspase 10	+	+	-
Caspase 12	-	-	-

Table 1. β-Lap exposures did not lead to caspase activation in NQO1-expressing breast cancer cells- Western blot analyses were performed to assess protease activation of specific caspases determined by loss of pro-enzyme forms with concomitant appearance of cleavage fragments representative of active enzyme forms. NQO1-expressing MDA-468-NQ3 cells were exposed to either a 4 h pulse of 8 μM β-lap or to continuous exposure with 1 μM STS or 10 μM TPT. Cells were then harvested at 24 h following STS or TPT treatments and at 6, 8, 10, 12 and 24 h after exposure to β-lap, experiments were performed at least twice.

Figure 1

A.


Apoptosis (%)
100
80
60
40
20
0
0
4
6
8
24
Time (h)

B.

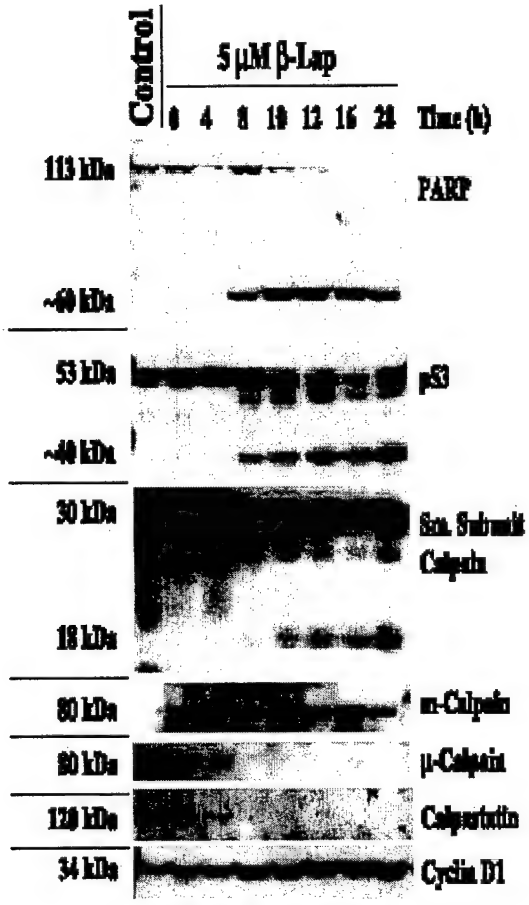
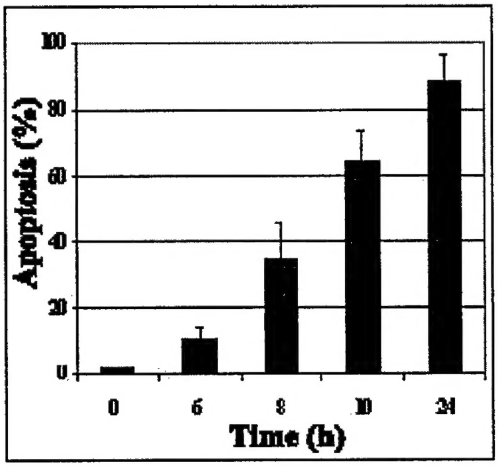

Control
5 μM β-Lap
0 4 8 10 12 16 24
Time (h)
113 kDa
PARP
~40 kDa
53 kDa
p53
~40 kDa
30 kDa
Sm. Subunit
Calpain
18 kDa
80 kDa
m-Calpain
80 kDa
μ-Calpain
120 kDa
Calpastatin
34 kDa
Cyclin D1

Figure 2

A.


Apoptosis (%)
100
80
60
40
20
0
0
6
8
10
24
Time (h)

B.

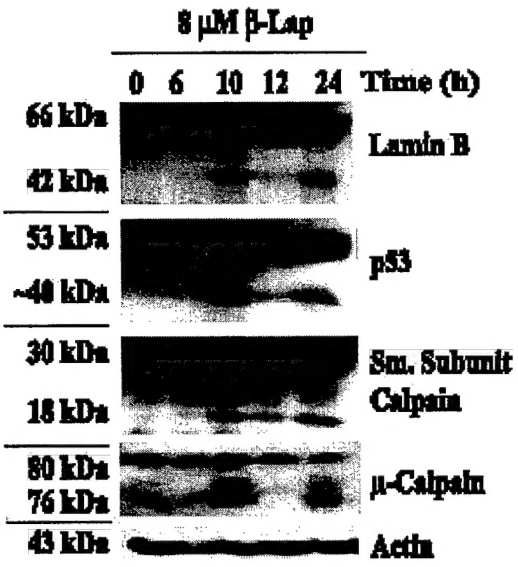

8 μM β-Lap
0 6 10 12 24 Time (h)
66 kDa
42 kDa
Lamin B
53 kDa
~40 kDa
p53
30 kDa
18 kDa
Sm. Subunit Calpain
80 kDa
76 kDa
μ-Calpain
43 kDa
Actin

Figure 3

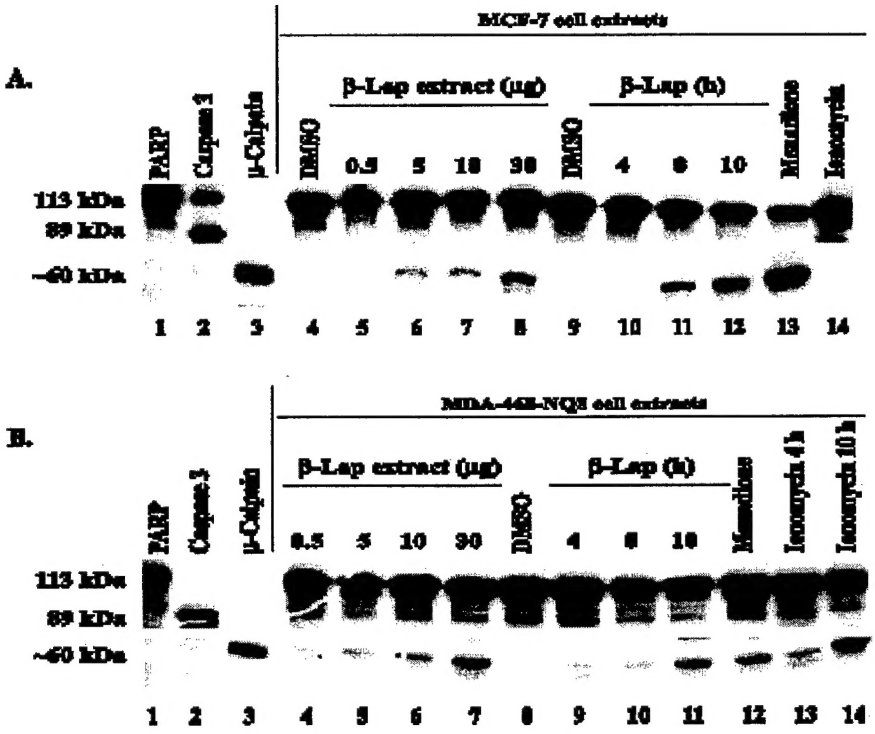

A.
MCF-7 cell extracts
β-Lap extract (μg)
β-Lap (h)
PARP
Caspase 3
μ-Calpain
DMSO
0.5
5
10
30
DMSO
4
8
10
Mitomycin
Ionomycin
113 kDa
89 kDa
~60 kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B.
MDA-468-NQ8 cell extracts
β-Lap extract (μg)
β-Lap (h)
PARP
Caspase 3
μ-Calpain
0.5
5
10
30
DMSO
4
8
10
Mitomycin
Ionomycin 4 h
Ionomycin 10 h
113 kDa
89 kDa
~60 kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 4

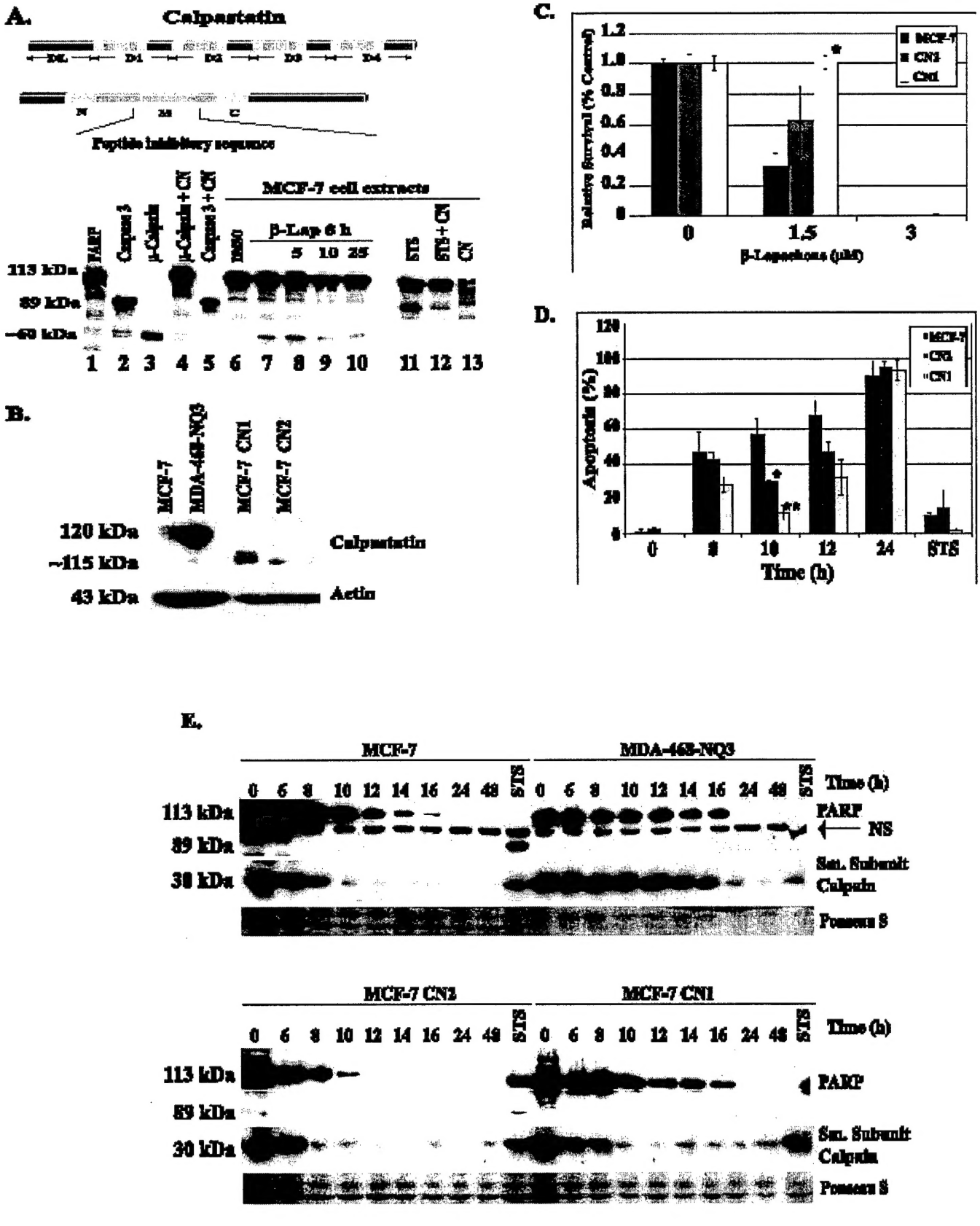
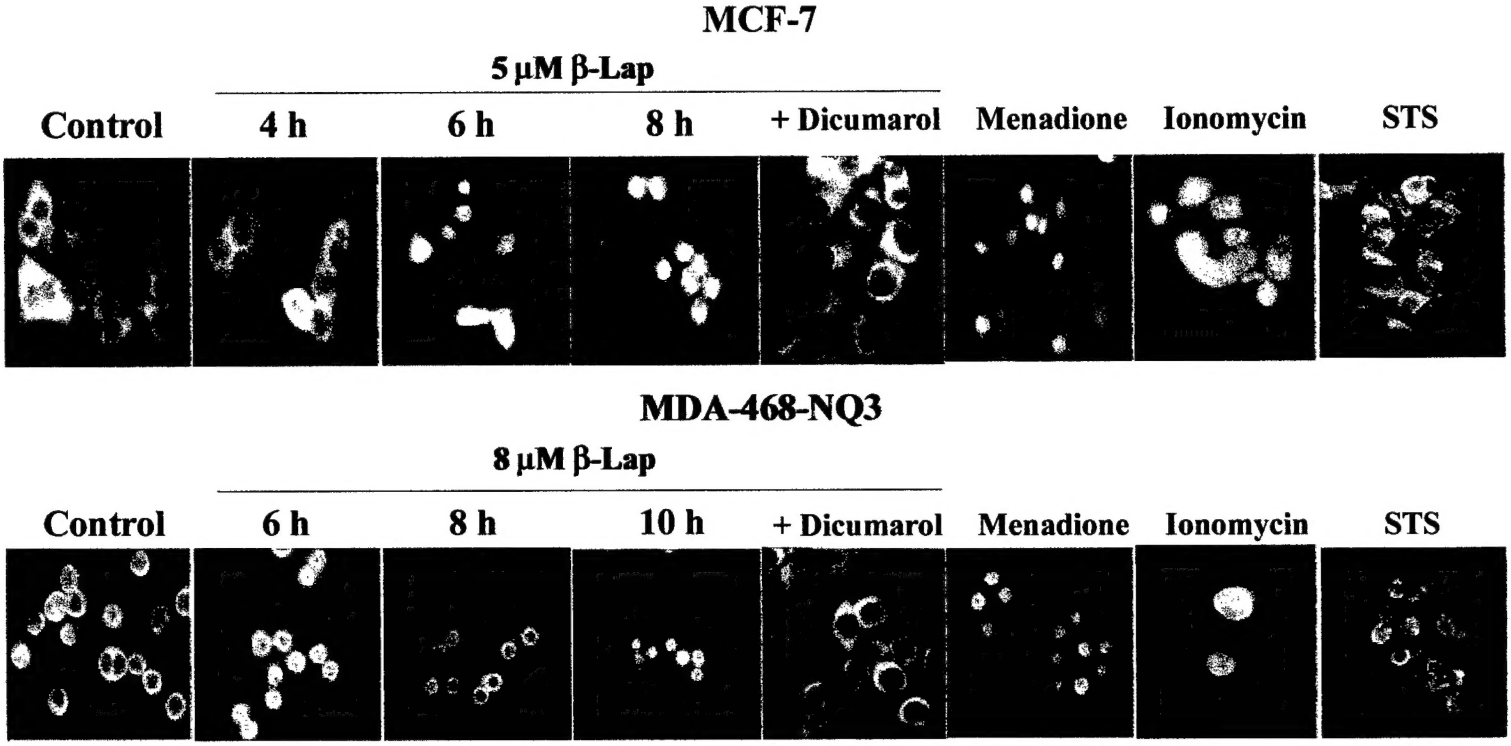
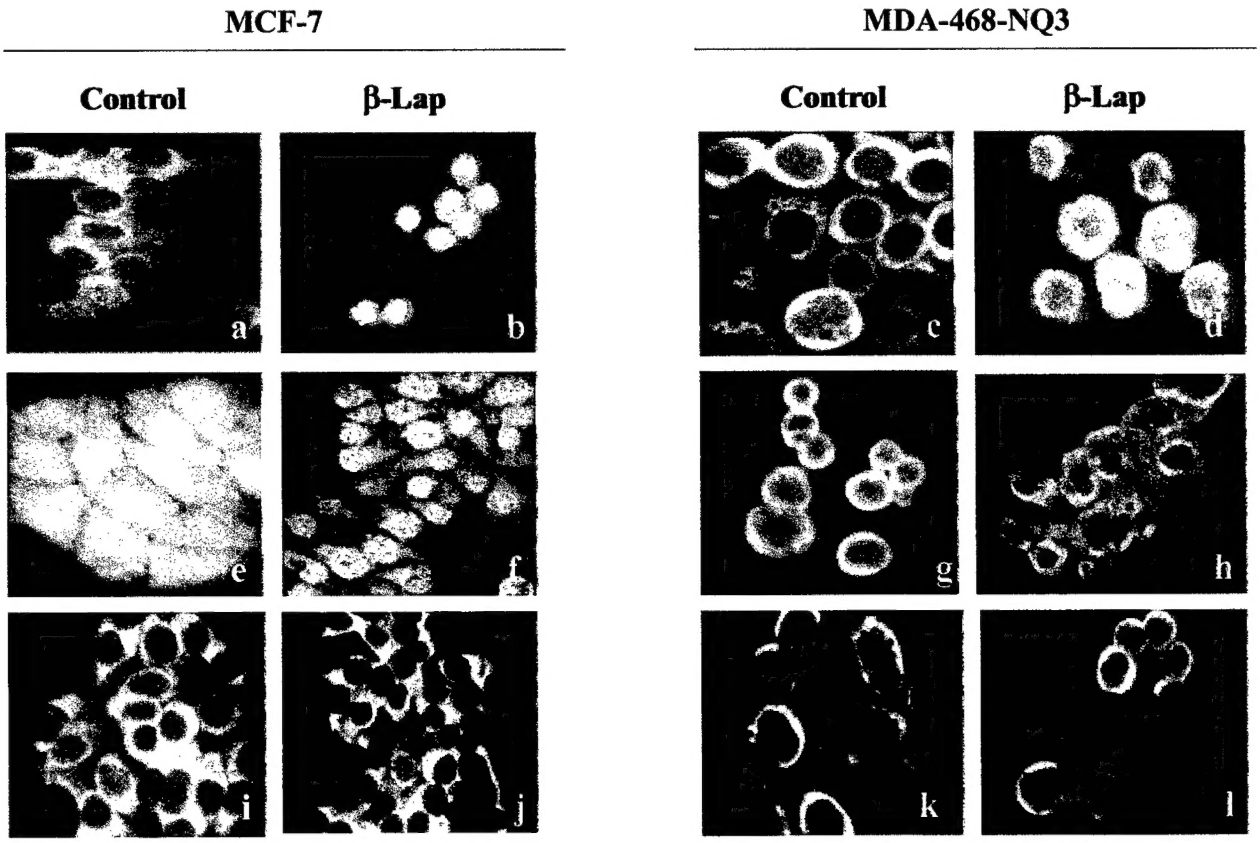

A.
Calpastatin
D0
D1
D2
D3
D4
N
28
C
Peptide inhibitory sequence
MCF-7 cell extracts
PARP
Caspase 3
μ-Calpain
μ-Calpain + CN
Caspase 3 + CN
DMSO
β-Lap 6 h
5
10
25
STS
STS + CN
CN
113 kDa
89 kDa
~60 kDa
1 2 3 4 5 6 7 8 9 10 11 12 13
B.
MCF-7
MDA-468-NQ3
MCF-7 CN1
MCF-7 CN2
120 kDa
~115 kDa
Calpastatin
43 kDa
Actin
C.
Relative Survival (% Control)
1.2
1.0
0.8
0.6
0.4
0.2
0
MCF-7
CN2
CN1
0
1.5
3
β-Lapachone (μM)
D.
Apoptosis (%)
120
100
80
60
40
20
0
MCF-7
CN2
CN1
0
8
10
12
24
STS
Time (h)
E.
MCF-7
MDA-468-NQ3
0 6 8 10 12 14 16 24 48 STS
Time (h)
113 kDa
89 kDa
30 kDa
PARP
NS
Sm. Subunit Calpain
Ponceau S
MCF-7 CN2
MCF-7 CN1
PARP
Sm. Subunit Calpain
Ponceau S

Figure 5

A.


MCF-7
5 μM β-Lap
Control
4 h
6 h
8 h
+ Dicumarol
Menadione
Ionomycin
STS
MDA-468-NQ3
8 μM β-Lap
Control
6 h
8 h
10 h
+ Dicumarol
Menadione
Ionomycin
STS

B.


MCF-7
MDA-468-NQ3
Control
β-Lap
Control
β-Lap
a
b
c
d
e
f
g
h
i
j
k
l